NEW YORK STATE ASSEMBLY

PUBLIC SERVICE LEGISLATIVE STUDIES PROGRAM

Stanley Steingut
Speaker of the Assembly

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The Microbial Degradation of Oil in Continuous Culture in Freshwater Ecosystems

Roy M. Ventullo

Prepared under the direction of Dr. Parmely H. Pritchard Associate Professor of Microbiology in the Department of Biological Sciences, State University of New York, College at Brockport, Brockport, New York 14420

This study was administered by the New York State Assembly Scientific Staff as part of its Public Service Legislative Studies Program which is supported by the Assembly, a grant from the Office of Intergovernmental Science and Research Utilization of the National Science Foundation to the New York State Assembly, and the New York Sea Grant Institute.

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The conclusions expressed are the independent results of the author and do not necessarily reflect views of the supporting agencies.

August, 1975

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Portions of the research implemented for the preparation of this request will be applied toward the partial fulfillment of requirements for a Master's Degree of Science in the Department of Biological Sciences. Parts of this report will also be directly used in the preparation of a thesis document which will ultimately be presented to this department for consideration and approval.

Parmely H. Pritchard

Parmely H. Pritchard, Ph.D.
Associate Professor

I. SUMMARY

This project has dealt with the development and testing of an experimental laboratory model for the microbial degradation of oil in aquatic ecosystems. The type of oil used in these studies was a light diesel oil with a boiling range of 230-300° C and a composition consisting of 25% normal paraffins, 50% cyclic paraffins and 20% aromatics. The model, which is a continuous culture system, allowed us to observe the fate of a mini-oil spill under quiescent conditions in a laboratory situation which closely mimics the environmental conditions of Lake Ontario. We have demonstrated the existence of a complex and very sensitive degradation process which is carried out by a diverse population of bacteria. The bacterial attack follows a consistent pattern of slow emulsification, dispersion , and chemical alteration of the oil. The complexity of the process, established herein, exemplifies the impracticability of using "seeding" procedures with commercial oil-eating bacterial preparations to deal with oil spills. We have verified that the disappearance of oil from the surface of a body of water does not by any means coincide with its complete degradation.

The oil degradation process in our system was readily altered by the presence of simple organic pollutants and not necessarily enchanced by the addition of detergents. We also have evidence to indicate that partially degraded oil may be toxic to aquatic organisms.

Our work further illustrates the preeminent importance of knowing not only how bacteria remove oil from sight but of how they affect the fate of the oil once it becomes dispersed in the water column.

II. BRIEF SYNOPSIS

The primary goal of our project has been to determine the environmental factors which affect and control the degradation of diesel oil by bacteria in freshwater ecosystems. Since bacteria are the ultimate disposers of oil in aquatic environments, we feel very strongly that a thorough knowledge of their degradation capabilities and the conditions which optimize their degradation process is essential to the creation and administration of oil pollution abatement programs by officials at all levels of public service.

Our use of continuous culture techniques has been an attempt to look at these oil degradation processes in a laboratory situation which mimics the conditions of a natural aquatic environment. Our system is analogous to the degradation of an oil slick under the quiescent conditions of a bay or enclosed harbor. We have designed a special culture vessel in which the oil floats on the surface of a water column and fresh, nutrient supplemented Lake Ontario water is passed continuously underneath the oil layer. The attack and subsequent removal of the oil by bacteria was studied by observing physical and chemical changes in the oil and by monitoring the composition and degradation potential of the bacterial populations.

Several major conclusions can be drawn from our experiments.

a) The oil degradation process is a very sensitive one which can be easily upset by certain environmental conditions. For example, it appears that a particular bacterial population is necessary for the initial attachment to and penetration of the oil layer before any significant degradation or dispersion of the oil can take place. The success of this initial step

appears to be a function of the inoculum and certain environmental conditions.

b) Degradation of the oil in our continuous culture system was very slow but the patterns of degradation agreed very closely with studies done by other investigators who have sampled oil directly from aquatic environments. A consistent observation in our studies was the relatively rapid physical dispersion of oil into the water column (that is, its visual disappearance) contrasted with the relatively small amount of actual decomposition which had taken place.

c) We have estimated that it would take approximately 50-60 days to completely remove, by bacterial degradation, a 2 mm thick layer of oil from the surface of a body of water under nonturbulent conditions. This would be roughly equivalent to the removal of two gallons of oil spread over a tenth of an acre of water surface. Under more turbulent conditions, the oil would probably disappear more rapidly through physical dispersion, but the extent of degradation cannot be estimated at this time.

d) Our results have indicated that the degradation of oil in polluted waters, i.e., the presence of readily degradable organic materials, may be much slower than in non-polluted waters. The addition of small amounts of dextrose or organic acids essentially inhibited degradation of the oil, presumedly because the bacteria found it to be a more palatable source of food.

e) The addition of detergents also interferred with the degradation of the oil again because the bacteria seemed to prefer to grow at the expense of the detergent rather than the oil.

f) The addition of commercial preparations of "oil-eating" bacteria had no effect on oil degradation. They were rapidly displaced by indigenous oil degradation populations in the lake water. The complexity and sensitivity of

of the oil degradation process that we have observed would tend to rule out any possibility of obtaining a practical preparation of "oil-eating" bacteria which would truly degrade the oil.

g) In initial toxicology studies, it appears that oil degradation products may be detrimental to the development and eventual hatching of fish embryos.

h) And finally, it would appear that the use of continuous culture techniques to study oil degradation is quite feasible and productive. Their similarity to natural aquatic situations makes our results more reliable and gives us information about oil degradation mechanisms which could not be obtained by any other technique.

From our studies it is clear that oil pollution abatement policies and oil cleanup procedures must be formulated around the potentialities and sensitivities of oil degrading bacterial populations if one is going to be at all certain as to the ultimate fate of oil in aquatic environments. We have therefore made several recommendations concerning oil pollution abatement (see pages 75-76 based on our knowledge of these bacterial activities. These recommendations include the use of mechanical dispersion and containment methods rather than the extensive use of detergents and the rejection of use of commercially prepared "oil eating" bacterial suspensions.

III. INTRODUCTION

A. The Need for Degradation Studies in Terms of Potential Legislative Action

With the present onslaught of a vast array of organic chemicals which are entering our aquatic resources it is becoming increasingly important to know the fate of these materials in the affected ecosystem. Undoubtedly the fate of organic pollutants is as diverse as the chemical nature of the pollutant itself but it is, nontheless, recognized that the metabolic activities of bacterial populations play a critical role in determining where the pollutants will ultimately residue or how fast the pollutant will eventually disappear.

Despite the fact that many microorganisms have been isolated in the laboratory which degrade a large variety of organic pollutants, such as oil or recalcitrant pesticides, surprisingly little is known about their actual fate in aquatic ecosystems. How, for example, do bacteria completely degrade a particular organic pollutant? Are the degradative mechanisms involved sensitive to environmental conditions? Are their degradative capabilities as efficient at low substrate concentrations as they are at high substrate concentrations. How fast do these processes operate under different conditions? And finally are the degradation products more toxic to the environment than the original pollutant itself? The answers to these questions are of particular importance because it is quite clear now, that the awareness of bacteria which can degrade a particular pollutant is not enough to allow us to predict its ultimate fate in an aquatic environment. Instead, as is evident from the work with "cometabolism" (27), it is essential that the entire bacterial population should be examined.

It is also clear that many officials at many different levels of administration and public service will eventually be forced to make decisions about the treatment of oil spillage. Such decisions require a knowledge of what will ultimately happen to the oil when it becomes dispersed in our lakes and streams. as well as our oceans. The absence of this knowledge, to date, makes these decisions very difficult and often unreasonable. For example, the Environmental Protection Agency is placing greater and greater restrictions on discharges into oceans and lakes without really knowing the capacity of aquatic environments to handle such discharges. This is a crucial point because EPA is setting the precedents upon which much of the state and local legislation for water quality standards and environmental impact policies will be based. It is clear, therefore, that the greater the data base, the more realistic and meaningful the resulting decisions will be.

B. Aspects of Oil Pollution

In the past 5-10 years large catastrophic oil spills such as the Torry Canyon, Santa Barbara, and Gulf of Mexico spills have aroused tremendous public concern and have made it clearly evident that our aquatic resources are becoming badly polluted with oil and petroleum hydrocarbons. Blumer (9) has estimated

aquatic resources are becoming badly polluted with oil and petroleum hydrocarbons. Blumer (9) has estimated an influx of 10^7 metric tons of oil per year due to shipping losses alone. Other losses, from sewage, motor oil, and incomplete combustion of fuels may add considerably to this loading.

Once oil enters the aquatic environment, it can cause enormous damage to the flora and fauna. Its physical presence can smother an animal, resulting in his death by asphyxiation or starvation. Plants can be damaged by disruption of oxygen transfer or alteration of other physiological processes.

Components of oil can also be chemically toxic to organisms in the environment. In general, the small molecular weight hydrocarbons, particularly the aromatic types, are the most toxic components of the oil. Benzene and toluene have been shown to be toxic to fish at between 10 and 40 parts per million (41). Higher molecular weight aromatic compounds, particularly pyrenes and dibentracene types, have been found to be carcinogenic to animal life in the sea (57).

Man is also adversely affected by oil pollution in the aquatic environment. Besides resulting in direct death of fish and other life, oil has been known to taint various commercial fish and shellfish making them unfit for human consumption. When beaches and surrounding waters become impregnated with oil, they are rendered unsuitable for swimming, boating, or fishing.

This recent awareness of the magnitude of oil pollution has been accompanied by a loud public outcry to cleanup the oil and "get it out of sight." Unfortunately, the methods for control and removal of oil spills are numerous and extremely diverse in principle. Many of the methods are largely untested in actual situations and when they are tested there is no assurance that different environmental situations can be handled with the same method. There are examples known where a particular cleanup procedure has damaged an ecosystem more than the oil itself (30).

The eventual fate of oil depends on two basic processes; dispersion and destruction. Dispersion is primarily a physical process involving, emulsification, sinking, evaporation, and absorption. The extent to which each of these factors affects the ultimate fate of the oil depends in good part on the type of oil at hand and the environmental parameters involved. In general, however, physical dispersion is only responsible for the removal of the oil from sight and does not infer destruction of the oil.

It is well recognized that bacteria are the primary natural disposers of oil and petroleum hydrocarbons in aquatic environments, and are ultimately responsible for ridding the environment of oil pollution (56).

Therefore, it becomes very important to know if the aquatic environment has the capacity to ultimately degrade oil into harmless byproducts and to know if the degradation process is complete under all environmental conditions.

C. Laboratory Studies of Natural Degradation Processes

Aquatic environments are characteristically open systems; they are not bound by walls or boundaries and there is free exchange of all parts of the environment with any other part at any time. This aspect of aquatic environments has largely been ignored in many laboratory degradation studies. As a result, degradation research has centered around the microbial activities occurring in closed containers or batch culture experiments, systems which have no equivalent in natural ecosystems. Most of the present information about the types of bacteria involved, the range of substrates attacked, and the mechanisms of oxidation, has originated from batch culture experiments. Attempts to use this information to predict the fate of oil or petroleum hydrocarbons in natural aquatic environments, however, has not met with success and generally created more questions than it has answered (20).

Part of the reason that this laboratory information cannot be readily extrapolated to a natural situation is because with these closed systems, the observed degradation is a function of time-dependent factors which are constantly decreasing or increasing at a rapid rate. As a result, during the course of an experiment, nutrient supply constantly decreased, metabolic end products and cells continually increase and many other parameters change unpredictably with time. Thus, the part of the bacterial population which is mediating the degradation process is the one which copes best with the conditions of the batch culture and does not necessarily reflect the influence of natural environmental factors on the degradation process.

In natural aquatic environments, or typically open type systems, drastic time-dependent changes are greatly minimized, although probably never completely eliminated. Because of this particular characteristic of aquatic systems, it is often desirable to study microbial degradation processes with continuous culture techniques since they are also open systems. Although continuous culture techniques are not an exact counter part with nature, they represent a technique which will permit greater extrapolation of the information obtained to the natural situation than does any other technique presently available. They also possess several other advantages:

a) They are based on controlled situations which are a function of a single growth limiting factor

b) They employ conditions in which factors affecting growth of a bacterium is time-independent

c) They allow a much greater latitude of experimentation on the factors which effect the degradation process

d) They permit one to do studies at concentrations of substrates and growth factors which are typical of natural aquatic environments

e) They have excellent potential for studying the degradation of the metabolic end products generated from the primary attack on the oil itself.

One of the major disadvantages of continuous culture is that they are highly selective; only the organism which grows the fastest is able to persist in a continuous culture system and all others are washed out. This situation is not strictly typical of a natural environment since organisms are only displaced but not eliminated.

Even so, the ecological studies which have been done with continuous culture systems have proven useful. Jannasch (18) for example, has shown that you can use continuous culture systems to enrich for bacteria which are specifically adapted for growth under particular environmental factors such as low substrate concentration, different types of substrates, changes in pH, etc. Many of the bacteria have proven to be very sensitive species which cannot be isolated by batch techniques. It is because of these reasons that we have chosen continuous culture techniques for this study.

D. Goals of This Study

It has been our objective over the past year to develop a laboratory model for the microbial degradation of diesel oil which can be used to generate much of the needed information about the fate of oil in aquatic environments. We have initially coordinated our efforts around the premise that the information generated in the laboratory must be directly applicable to natural situations.

Our development of an experimental laboratory model for the study of the microbial degradation of oil spills in freshwater has been accomplished thru the use of continuous culture systems in which oil is layered on the surface of a water column and continuously exposed to fresh media. The system is somewhat similar to the natural degradation of an oil slick under quiescent conditions.

The goals of our work were those originally set forth in the project proposal and they are as follows:

a) to determine the mechanisms by which crude oil is degraded by bacteria in freshwater continuous culture systems.

b) to determine the environmental factors which effect the degradation rates and mechanisms.

c) to determine the toxicity of crude oil degradation products on selected bioassay systems.

d) Plan of Procedure
   1) Freshwater continuous culture systems will be set up in which fresh media and bacteria can be continuously passed underneath an oil layer.

   2) Gas and liquid chromatographic analysis of an oil will be worked out in order that chemical changes in the oil can be monitored.

3) Degradation of a diesel oil will be followed in continuous culture by monitoring chemical changes in the bacterial populations using standard culture techniques.

4) Environmental factors, such as inorganic nutrient concentration, polluted versus clean water, temperature, particulate matter, detergents, etc. will be studied to determine their effects on the degradation process. Field studies will also be carried out using Brockport's scientifically equipped houseboat. This will allow fresh lake water for different stations to be continuously pumped into our growth systems.

IV. LITERATURE REVIEW

A. Composition of Oil

Petroleum is a highly complex organic mixture consisting largely of hydrocarbons of several different molecular types, the three principle classes being: alkanes, cycloalkanes, and aromatics. Alkanes, hydrocarbons which contain only single bonds, account for a large proportion, 36% to 65% of the total weight of most petroleum distillates (49). The most abundant series, the normal or straight chain alkanes, contain from one to seventy-eight carbon atoms. Iso- or branched alkanes, especially the 2, 3, and 4-methyl alkanes occur in relatively high concentrations (10 to 15 weight percent) and individual members up to C40 have been isolated from crude oil (47). Highly branched compounds are not as common in crude oil, although three particular compounds, pristane (2,6,10,14-tetramethylpentadecane), phytane (2,6,10,14-tetramethylhexadecane), and farnesane (2,6,10-trimethyldodecane) are found in concentrations ranging from 0.7 to 1.8 weight percent.

The cylcoalkanes which contain at least one saturated ring structure, although they are not as prevalent as the normal and iso alkanes, less than 15 weight percent total, are present throughout the entire boiling range of crude oil (49).

Aromatic compounds, on the other hand, contain at least one unsaturated, benzene component in their structure. Benezene, phenanthrene, and anthracene as well as many tetra- and penta- ring compounds can be found in this aromatic series. The predominant aromatics in crude oil, however, are the alkyl-substituted benzenes and napthalenes. Individual compounds which have been isolated are 1-methyl-3-ethylbenzene, 1,2,4-trimethylbenzene, and 1-, 2-, and 3-methylnapthalenes.

There are also many sulfur, nitrogen, and oxygen containing compounds found in varying amounts in most oils (49). Sulfur compounds usually comprise one to two weight-percent whereas oxygen and nitrogen compounds are usually found in concentrations of less than 0.9 weight-percent.

B. Physical and Chemical Factors Affecting the Disappearance of Oil

Auto-oxidation, a chemical reaction which liquid hydrocarbons undergo in the presence of gaseous oxygen and light, is one of the first chemical changes which occur in oil spilled in the environment (37). Organic hydroperoxides appear as the main product, and in the presence of the sulfur compounds and metals, usually found in oil, these oxides are converted to ketones, acids, and aldehydes (42). Although auto-oxidation is a continuous process, it is extremely slow and does not contribute significantly to the ultimate fate of the oil.

Sinking, in contrast, is a very rapid process which removes large amounts of oil from the surface and deposits it in the sediments. (14) It is generally

a process whereby the oil becomes impregnated with debris (e.g. clay particles, bacteria, plankton, etc.) giving a brown soft mass which is heavier than water. Once in the sediment, microbial degradation, dissolution, evaporation, and auto-oxidation slow down and may even be completely inhibited. Thus, sinking may actually increase the residence time of oil in the environment,

Dissolution and evaporation are responsible for loss of many volatile components (boiling point 300°C) of oil (56) even some of the higher molecular weight water-soluble polar components (38). It has been shown that dissolution and evaporation have been responsible for the loss of up to two-thirds of a Venezuelan crude oil within a few days in the ocean (21).

The use of mechanical and chemical clean up procedures to remove the polluting oil have met with varying degrees of success. Absorbents, such as straw, clay, and polyurethane have been used to collect oil. These are useful in that they facilitate removal of the oil from the shore but they must be used immediately before the oil has a chance to spread over a large area. Oil herders both chemical and mechanical have been used to contain the oil to a smaller area, but have met with only limited success (55, 7), due to rough seas and acute structural problems. Suction pumps have been tried but are only useful when the oil layer is several inches thick (14).

Solvents and emulsifiers have been used to a much greater extent than the above mentioned processes (55). Addition of these chemicals causes the oil to spread over a much larger surface area. This then increases the amount of dispersion within the water column and thereby increases the degradation of oil by microorganisms. Unfortunately, many of these chemical dispersants and emulsifiers are toxic or bacteriostatic in even a few parts per million (56) and thus have limited value in clean up procedures.

C. Role of Bacteria in Physically Removing Oil

The emulsification of oil is probably one of the first processes mediated by oil degrading bacteria. It is simply a process whereby the oil is broken up into a permanent suspension of tiny oil droplets. It has been commonly observed in many degradation studies and there are indications that the emulsification may be the result of a proteinaceous-type compound elaborated by bacteria. Since it appears that bacteria associate themselves with oil droplets as a means of attacking it (24), emulsification, which produces more surface area, is a very important initial step in the microbial degradation of oil.

In addition to emulsifying oil, it has recently been found that bacteria are capable of sequestering oil and individual petroleum hydrocarbons (15, 18, 19). In the sequestering process, the hydrocarbons or oil is taken up and stored in inclusion bodies within the cells. Finnerty et al (19) have shown that under natural conditions bacteria were able to sequester crude oils, tar residues, and n-alkanes, indicating that the sequestering process

may play some role in the removal of oil from the surface of a body of water.

D. Microbial Hydrocarbon Degradation

Although it has been well documented that fungi are capable of degrading oil and hydrocarbons (34. 1), it appears that bacteria are the prime degraders in the aquatic environment because of their competitive abilities. It is clear from many studies that a large number of bacterial species are capable of degrading hydrocarbons and participating in the degradation of oil (56). Hydrocarbon metabolism is certainly not a unique aspect of microbial populations and in fact bacteria isolated from different ecosystems for varying reasons often show hydrocarbon degradation capabilities (22).

In terms of substrate specificities, individual bacteria attack only a narrow range of hydrocarbons. Bacterial populations, however, demonstrate an impressive array of hydrocarbon oxidative mechanisms. As has been established numerous times, n-alkanes are utilized with greater frequency and rapidity than other hydrocarbons in liquid cultures (34). There is apparently a specificity within the n-alkane series as well. Alkanes of 10 carbons or less are not degraded as frequently, or by as many bacterial species, as those hydrocarbons with carbon chain lengths of ten or twenty-four (22, 44).

Branched alkanes are fairly resistant to microbial degradation in comparison to n-alkanes, although this may be a reflection of the fact that they have not been studied as extensively. There is, however, no question that they can be broken down by a variety of different bacterial species.

McKenna (39) has shown that microbial assimilation of alkanes was affected by the degree of branching and the size and position of the branches. For example, if properly placed, multiple methyl branches did not render an alkane unpalatable to the species tested. However, a quaternary carbon atom (neopentyl group) occurring at the end of an alkane molecule, resulted in a structure quite resistant to microbial degradation.

The cycloalkanes are very difficult hydrocarbons to breakdown and no one to date has isolated an organism which will use cyclohexane as a sole source of carbon and energy. Even the addition of short chain n-alkyl substitutions does not render them susceptible to microbial oxidation (43). Beam and Perry (5) however, have shown that several strains of bacteria were able to degrade long chain n-alkyl substituted cycloalkanes. Furthermore, they noted a greater probability of ring cleavage when the side chain contained an odd number of carbons.

The aromatic hydrocarbons represent the most diverse group of compounds and are probably the most recalcitrant fraction of the oil. However, aromatic hydrocarbon oxidation is well established especially for the benzene and naphthlene related hydrocarbons and the primary differences in degradability depends on the type of functional group substitution (23, 35). Methyl group substitution at certain positions has been shown to slow up degradation and even completely inhibit it. On the other hand, the substitution of various alkyl groups on a benzene ring renders the resulting compound more susceptible to degradation (39, 19).

Another aspect of hydrocarbons utilization which will strongly affect the degradation of oil in aquatic ecosystems is the phenomenon of co-metabolism (27, 46). Co-metabolism is the process by which a microorganism can oxidize a substrate which cannot or will not be used as an energy source. Many researchers have shown that certain hydrocarbons which will not suffice as a source of carbon and energy for isolated organism are in fact oxidized when mixed with soil especially if some other readily utilizable substrate is supplied (46). Beam and Perry (8), for example, have suggested that recalcitrant cycloalkanes may be degraded via co-oxidation while growing on other hydrocarbon substrates.

E. Microbial Degradation of Oil

There has been a relatively large number of studies on the degradation of oil by bacteria, almost all of these involving batch culture types of experiments, and it is now clear that there are certain trends which are routinely encountered. (32, 36, 10, 29). Beside the initial emulsification step described above, bacteria invariably attack the normal alkanes first. In a gas chromatographic profile the n-alkanes usually represent the most predominant peaks and these peaks are always the first to disappear during the degradation. Oils, in fact, which are low in saturates show a much slower rate of degradation (54).

In most oils, pristane and phytane (branched alkanes) are also present in high concentrations but they invariably take much longer to be degraded at least relative to the n-alkane. However, it is only a short lived method since, after sufficient incubation the pristane/phytane peaks also disappear (29, 45). The cycloalkane and aromatic fractions are the most recalcitrant fraction of the oil since in many batch culture experiments these fraction never do seem to disappear even after extensive incubation. This result is both a function of the cultural conditions and the sensitivity of the analytical techniques. In natural situations however, these recalcitrant fractions do disappear although there is no convincing evidence that they are actually degraded. In the laboratory, however, one probably immediately selects out for alkane utilizing bacteria eventually displacing those organisms capable of degrading these more complex fractions.

The time course involved in these degradations is highly variable. Generally, in the laboratory the n-alkanes are entirely degraded in 20-40 days whereas with those small numbers of experiments done in the field it may take 1-3 months to degrade the saturates and as long as 12-15 months to completely degrade the entire amount of oil present (10).

F. Environmental Factors Affecting Oil Degradation

There are various interrelated parameters which influence the microbial degradation of oil in the aquatic environment. Generally speaking, most hydrocarbon utilizers require free dissolved oxygen. ZoBell (58) for example, has determined that the biological oxygen demand of Barataria Bay bottom samples inoculated into an oil-mineral salts media was three to four

milligrams per liter. Thus, the complete oxidation of 1 mg of hydrocarbon to carbon dioxide and water required approximately 4 mg of oxygen. Partial oxidation of the hydrocarbon, involving the incorporation of molecular oxygen to form alcohols, acids, aldehydes, or esters, requires much less oxygen than does the complete oxidation to carbon dioxide and water. In situations where the organisms are in contact with the normal atmosphere, as at an air-water-oil interface, the supply of oxygen is quite adequate. Oxygen can become a limiting factor, though, in areas of intense microbial activity below the water's surface, particularly in bottom sediments.

There is still a great deal of controversy over whether bacteria can oxidize and degrade hydrocarbons under anaerobic conditions. Several investigators have reported the slow disappearance of oil in the complete absence of oxygen, using sediment samples (58, 12) but the actual mechanism of degradation has never really been fully clarified. Since sulfate and nitrate can serve as alternate electron acceptors, there would appear to be no problem in degrading a hydrocarbon once it was initially oxidized. However, this initial oxidation step is the most troublesome point since it has classically been a mechanism involving molecular oxygen. Despite the fact that Kallio (40) has argued that primary oxidation under anaerobic conditions is thermodynamically impossible, several lines of evidence now indicate that water can be added over a double carbon moiety thus producing an alcohol derivative of an alkane without the use of molecular oxygen (40). Whether this is a common type of reaction or not still remains to be established. Regardless, it is well established that the deposition of oil in an anaerobic environment drastically slows down the rate at which it is degraded by bacteria.

Temperature, also afffects oil degradation rates and there is now considerable evidence to indicate that temperatures below 10°C slow degradation substantially (1). Westlake and his colleagues (54) have presented preliminary evidence demonstrating the existence of different degradative mechanisms and different population compositions of oil degrading bacteria at 4°C. Of particular interest is the apparent decreased rate of aromatic degradation at these temperatures but no significant effect on the n-alkane utilization.

Nitrogen and phosphorous are of course required for oil degradation and they are probably the first factors to become rate limiting in the natural environment (56). For example, Atlas and Bartha (4, 6) reported the addition of either nitrogen or phosphorous to sea water did not stimulate biodegradation or mineralization (complete oxidation). The addition of both, however, increased degradation by 79% and mineralization by 42%. Experimental evidence of Jobson et al (30) has shown the addition of urea-phosphate to oil-impregnated soil, significantly increased the utilization of both the saturate and the aromatic fractions of the oil. The requirements of an oil utilizing bacterial population, then, can be supplied by a few parts per million nitrogen and phosphorous.

The presence of low concentrations of organic material may promote the growth of hydrocarbon utilizing organisms by providing needed co-factors such as vitamins or amino acids. However, Atlas and Bartha (5) reported the utilization of petroleum by two marine isolates was inhibited by the presence

of fatty acids, particularly short chain ones. It is feasible that catabolite repression by glucose or other organic compounds may have an effect on oil degradation but this has not been determined.

C. Methods of Oil Degradation Analysis

One of the big problems in oil degradation studies has been the lack of good analytical methods for monitoring the degradation process. Since so many different methods have been used, it is often difficult to compare results. Many are also limited in value, either because they are not quantitative enough or because they do not show the degree or completeness of degradation.

Column chromotography has been somewhat successful in analyzing changes in oil caused by microbial degradation (11, 15, 32). Silica gel and silica gel-alumina dual phase column chromotography have been used to separate alkane and aromatic fractions of crude and diesel oils (29, 32). Once separated, bacterially mediated changes in the two fractions can then be determined by gravimetric means (29) or gas chromatography (29, 11).

Used in conjunction with liquid chromotography, gravimetric analyses allows an absolute determination of the extent of degradation of the alkane and the aromatic fractions of the oil by comparing weight loss relative to a stock oil. Both Kator (32) and Jobson et al (29) have obtained significant information using this method. Gravimetric measurements however are not qualitative and cannot be used to determine the utilization of individual components of a mixture.

Gas chromatography itself or in conjunction with liquid chromatography can be used for both qualitative and quantitative changes brought about by microbial action on oil. The separation of components in a very small sample can be obtained very rapidly and with a high degree of resolution. Qualitative analysis is based on comparison of the retention time of the unknown compound with that from a known hydrocarbon, under identical conditions (25).

Various workers have used gas chromatography to study oil degradation and it is now considered a standard technique. Soli and Bens (48) for example, have determined the extent of utilization of an artificial oil by analytical gas chromatography. Walker and Colwell (53) have recently used liquid and gas chromatography to determine the microbial utilization of a motor oil and an artificial oil in laboratory experiments. Gas chromatography has also been found to be extremely useful in field studies also, as exemplified by work of Blumer et al (10) and Jobson et al (29).

There is however one problem which has become apparent, since gas chromatography has been used to analyze complex oil mixtures. Researchers have been unable to resolve the gas chromatographic profile which is left in the residue of degraded oil. Mass spectral data have indicated that the undegraded envelope is comprised of one to six ring cycloalkanes and various polyaromatic compounds which cover a large boiling range (53).

Simple visual observation of a gas chromatographic profile is generally not enough to verify actual chemical change brought on by bacterial degradation. Instead quantitative relationships between certain components in the oil must be evaluated. The peak height ratios of n-C17/pristane and n-C18/phytane have been established to be a valid indication of compositional changes occurring in the oil, and also appears to be a sensitive indicator of bacterial degradation of oil (11). The method is based on the recalcitrant nature of the iso-alkanes, pristane and phytane. Since they are much less susceptible to microbial attack, the n-alkane ratio, a preferential decrease in the concentration of the respective n-alkane is indicated, whereas an increase in the ratios would indicate a preferential utilization of the iso-alkanes. One drawback with this technique, is that if both pristane and phytane are degraded and there are reported cases of branched alkane oxidizing bacteria (45) simultaneously along with their respective n-alkane, the ratios would indicate no degradation.

Thus, in order to further substantiate the results obtained, using the peak height rates method, peak-envelope ratios were also calculated. The ratio of the peak height to the envelope depends on the recalcitrance of the envelope components and this has been reported in the literature to be the case (29, 53). The ratio of the peak height above the envelope to the envelope itself would then give us another method to determine the extent of degradation of the oil by bacteria.

V. MATERIALS AND METHODS

A. Continuous Culture Techniques

The basic design of our continuous culture apparatus allowed us to study the degradation of diesel oil in an undisturbed two phase system (see Fig. 1A). Oil was floated to the surface of a water column allowing the continual flow of media beneath it. Flow rates were controlled by a peristaltic pump (Harvard Apparatus, Model 1203). A glass 18 mm tube in the center of the growth vessel (which was part of the aeration system), prevented mechanical dispersion and emulsification of the floating oil. Silicon tubing was used wherever flexible tubing was needed as this was resistant to bacterial contamination and physical changes due to hydrocarbons.

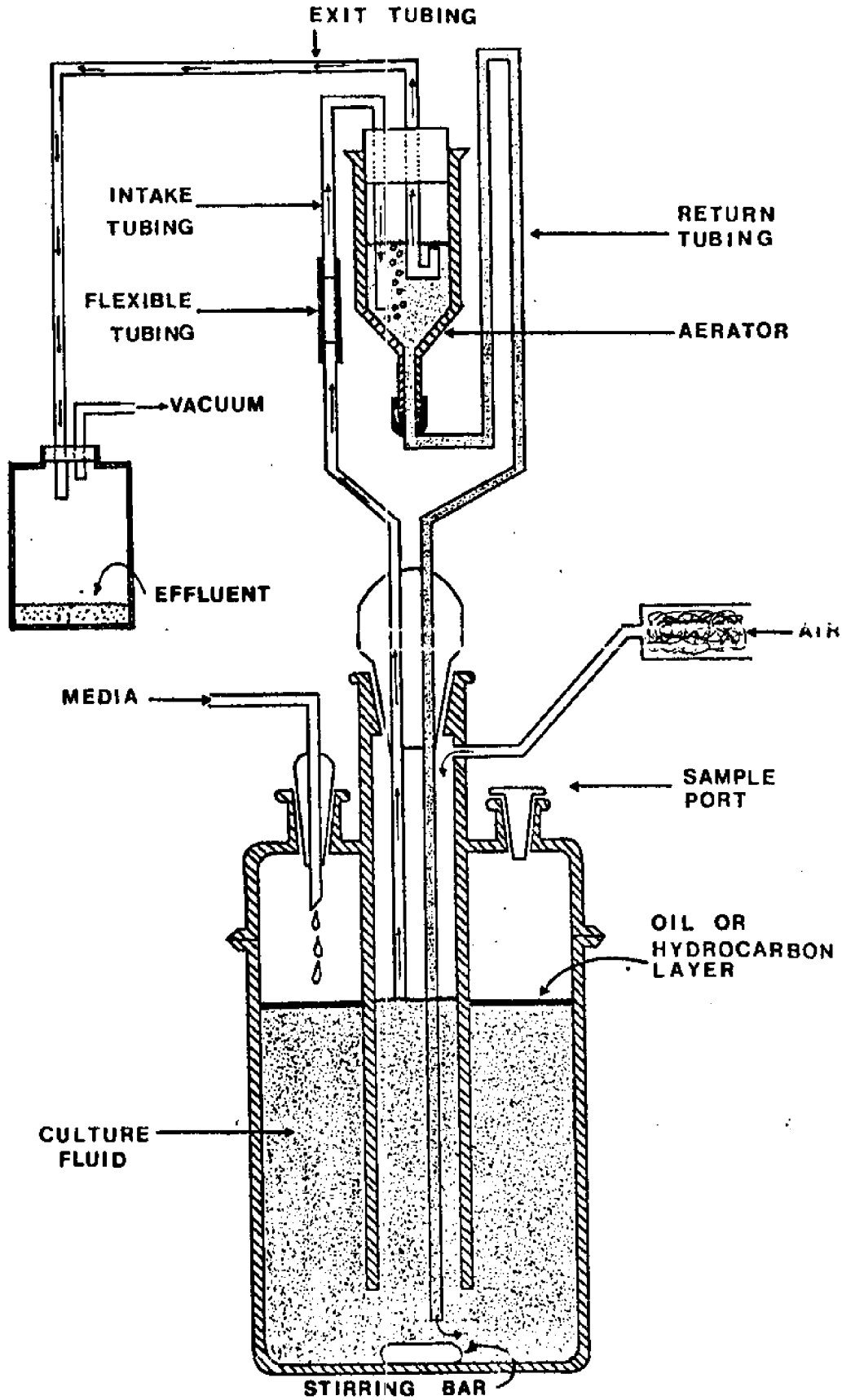
The aeration system was based on the use of a constant vacuum (drawn by a mechanical pump) to suck filtered air and culture fluid up into the aerator. The aerator was initially filled by pinching off the flexible portion of the intake tubing so that liquid was sucked up into the aerator via the return tubing. This procedure completed a siphon in the return tubing so that once the intake tubing was reopened, liquid was continually circulated and exited via this siphon. During the start-up procedure, the sample port was left open until the siphon was complete. A reservoir of culture fluid was continually maintained in the aerator tube by virtue of the constant vacuum. The volume in the culture vessel was maintained at 250 mls using a u-tube in the aerator.

Sampling was accomplished by first clamping off the overflow and intake tubings and then removing samples thru the sample port or from the inside of the center tube. This latter procedure could be done without breaking the siphon or disturbing the oil layer. By simply removing the clamps, the aeration process again commenced by itself.

All samples of water used to inoculate the continuous culture vessels were obtained from Lake Ontario at Hamlin State Park, Hamlin, New York. Samples were taken in 15 l Nalgene carboys and were used immediately upon return to the lab or stored up to three weeks at 10°C.

The medium used in the resevoir of the continuous culture systems contained 100 mg Na_2HPO_4-KH_2PO_4, pH 7.2 and 1C mg of each NH_4Cl and $MgSO_4$ per liter of medium. For initial unsupplemented oil experiments, salts were added as stock solutions to 14 l of distilled water which had been steam sterilized for 6-8 hours. In experiments to determine the effects of amendments on the degradation rates, soluble carbon sources were added to the resevoir at 0.1% concentration. In these experiments where hexadecane was the added carbon source, 1 ml was added to 7 ml of oil. This mixture was added to the culture vessel in place of the unamended oil.

Figure 1A - Continuous Culture System Used in Oil Degradation Studies



To start a continuous culture experiment, an inoculum of 250 ml of Lake Ontario water was placed in the culture vessel and amended with 25 mg of Na_2HPO_4-KH_2PO_4 and 2.5 mg of NH_4Cl and MgSO. Eight milliliters of topped diesel oil was added through the sampling port. The system was incubated at room temperature for 12-15 hours as a batch culture (no media inflow). The flow of media was then commenced and continued at a dilution rate of 0.05 hr^{-1}.

For each experiment a sampling schedule was set up so that the oil and bacterial populations were analyzed at ninety-six hour intervals. At each sampling period the following procedure was carried out:

a) Sixteen hours before any samples were taken, the catch flask (containing effluent) was emptied and washed.
b) An effluent sample was taken by collecting all the fluid which had flowed into the catch flask over a sixteen hour period. This sample was immediately extracted to recover any oil present and subsequently prepared for chemical analysis (see below).
c) An oil layer sample was taken by touching a disposable capillary pipette to the oil layer. Approximately 50 µl of oil would move into the pipette by capillary action. This oil was then washed out of the pipette with benzene-pentane solvent and subsequently prepared for chemical analysis (see below).
d) A culture fluid sample, for analysis of the bacterial populations, was taken by placing a sterile pipette into the center well of the continuous culture system and removing 1.0 ml of fluid. This sample was then immediately diluted and the cell numbers were determined by standard quantative spread plate techniques using Standard Methods agar (Difco). Plates were incubated at room temperature for up to fifteen days before examining. Isolates were distinguished entirely by colony morphology and were stored on Standard Methods agar slants at 10°C. The criteria for predominance was based on the colonies which made up the largest percentage of the total bacterial population.
e) Any physical changes in the oil were also recorded at this time.

In sequential continuous culture experiments, a second and third vessel were connected in series to the primary vessel as shown in Figure 1B. In this way the effluent from one vessel serves as the nutrient source for the second vessel. The volume of these vessels was 1:3:9.

The primary vessel of the sequential continuous culture system was then inoculated and incubated as indicated above. The second and third vessels were empty at the commencement of the experiment and were eventually filled with effluent from the first vessel.

Each of the vessels in the sequential system were sampled as described above. For oil analysis, approximately 200 mls was removed from either the second or third vessels and then this loss in volume was replaced with 200 mls of sterile Lake Ontario water.

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e) Any physical changes in the oil were also recorded at this time.

B. Determination of Substrate Specificity of Bacterial Isolates

Because of the insolubility of hydrocarbons in aqueous media, it is very difficult to determine which types of hydrocarbons are attacked as carbon and energy sources. The use of agar media under hydrocarbon atmospheres is commonly employed but it is often difficult to get good growth with this method. Liquid culture, in which hydrocarbons are dispersed as small droplets by mechanical agitation, has also been extensively used but here again it is some times difficult to determine if no growth means lack of the appropriate degradative pathway or inhibition of growth due to toxicity, smallness of inoculum or lack of some other growth factor.

To circumvent these problems we have done substrate specificity tests in liquid culture using large inoculums of log phase cells in media supplemented with sodium acetate as well as with hydrocarbon being tested. Each purified isolate was first grown in shake flasks containing 100 mls of GYP medium (1.0% glucose, 1.0% peptone and 0.1% yeast extract) until heavy turbidity was obtained. Ten mls of this culture was transferred to a large bubbler flask containing 300 mls of acetate minimal salts medium (NH_4Cl, 1 mg/l; Na_2HPO_4, 2 gm/l; KH_2PO_4, 0.8 gm/l; NaCl, 0.1 mg/l; $MgSO_4$, 0.04 gm/l; $FeCl_3$, 2.0 mg/l; Na acetate 20 gm/l) and incubated for 48 hours at room temperature. The cells were then harvested and washed twice, asceptically with 0.01 M sodium phosphate buffer, pH 7.2. On the second washing, the cells were concentrated three fold. Ten mls of this washed concentrated cell suspension was used to inoculate shake flask containing the hydrocarbon to be tested and acetate minimal salts medium containing only 2 gm/l sodium acetate rather than the original 20 gm/l. The initial optical density of these cultures was about 0.1 (420 nm). All liquid hydrocarbons tested were used at a concentration of 0.2 mls per flask (screw capped flasks were used for volatile hydrocarbons). Solid hydrocarbons were used at a concentration of 0.2 gm per flask.

Each of the hydrocarbon flasks was incubated on a shaker and optical density readings were recorded each day. In flasks where hydrocarbons were nontoxic, growth immediately commenced and continued until the acetate was consumed. If the isolate could use the hydrocarbon present for carbon and energy, then growth continued (measured by changes in optical density) but usually at a slower rate. If the isolate could not use the hydrocarbons, then the optical density remained constant, in many cases for as long as ten days.

C. Chemical Analysis of the Oil

Diesel oil used in this study was obtained from the commissary at SUNY Brockport, Brockport, New York. The oil was topped by placing one liter of the oil in a tared beaker and exposing it to forced draft conditions at 37°C. This process was continued until the rate of weight loss was negligible. This process removed volatile materials up to and including normal dodecane, leaving approximately 65% of the weight of the original oil. The topped oil was then filter sterilized twice through 0.45 μ Millipore filters and stored in sterile ground glass bottles. This sample of oil was used for all experiments.

The analysis of compositional changes in the oil during the degradation process was accomplished either by sampling oil directly from the oil layer itself, or by extracting residual oil from effluent samples, at the same time the oil layer was sampled. Approximately 0.2 ml of oil was asceptically removed with the capillary pipet at each sampling time and placed in a 2 x 10 mm conical tipped centrifuge tube containing 2 ml of distilled water. This solution was extracted twice by shaking with 15 ml of dry pentanebenzene solvent (1:1 by volume). The organic phases were then combined and concentrated in a 3 x 12 mm round bottom test tube.

To sample the effluent, 200 ml of culture effluent was collected and twice extracted by shaking with 25 ml of the same solvent in a 250 ml separatory funnel. The organic phase in these extractions were saturated with tiny air bubbles interdispersed in a white emulsion. In order not to contaminate the extracted oil with this emulsion, the solvent layer was carefully decanted into a 3 x 15 mm round bottom test tube, leaving the emulsion in the funnel.

All extracts were concentrated to approximately 0.5 ml under forced air conditions in test tubes suspended in a 32°C water bath. These concentrates were placed in 1.1 ml conical tipped storage vessels (Bellco Glass) along with 0.5 ml solvent rinse of each test tube. These solutions were further concentrated by a forced air draft. If visual examination of the concentrate demonstrated no further loss of volatile solvent, the samples were sealed with teflon or silicone lined caps. These concentrated samples were then analyzed by gas chromatography. The effluent samples consisted of culture fluid which had passed out of the continuous culture vessel into a special catch flask over a 16 hour period.

Gas chromatographic analyses were carried out using standard high temperature non-polar silicone columns. Column packing material was prepared by dissolving 0.15 g of OV-1 methyl silicone (Applied Science Laboratories) in 15 ml of analytical grade toluene, overnight. This solution was added dropwise to completely wet, but not soaked 5 g of Gas Chrom Q (Applied Science) in a 25 ml beaker. The toluene solvent was then evaporated at 40°C while gently agitating the mixture by hand.

Two ten foot stainless steel columns (1/8 inch I.D.) were cleaned with successive 300 ml volumes of hot detergent hot water, distilled water, and benzene and finally dried by passing thru a bunsen burner flame. Columns were packed by intermittent vibrating with a Vibro-Graver (Burgess) and plugged with glass wool. Columns were conditioned at 325°C for 48 hours while being purged with a small volume of helium gas.

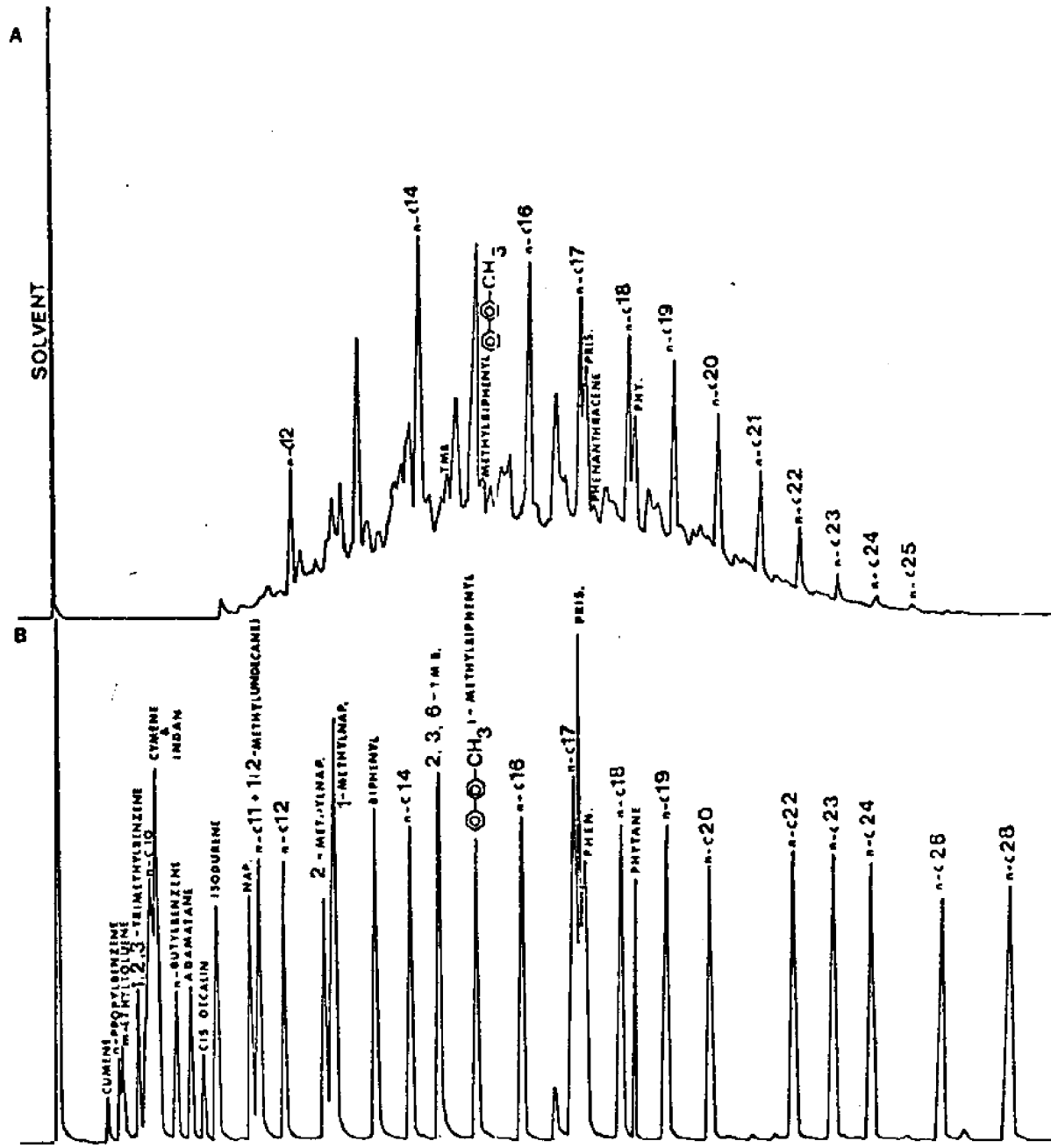
A Hewlett-Packard Model 5750 gas chromatograph equipped with a dual flame ionization detector was used in all analyses. The instrument was programmed as follows: linear temperature program 60-670°C at a programing rate of 4°C per minute; injection port temperature, 300°C; flame detector, 300°C; helium carrier flow rate, 20.0 ml/min; hydrogen flow rate, 12.5 ml/min; air flow rate 350 mg/min.

Benzene or pentane was used as a solvent for injection as they passed through the OV-1 column well in advanced of other compounds.

Characterization of the oil chromatographs was carried out by comparison with a standard hydrocarbon mixture (Figure 2). The standard mixture was prepared by adding 30 mg of solid or 30 µl of liquid hydrocarbon to 200 µl of benzene solvent. Samples were injected into the gas chromatograph and retention times of the hydrocarbons determined. The final mixture contained 30 resolvable components and is referred to as ARTOIL in the text. It was possible to identify all of the n-alkanes series, two isoalkanes, pristane and phytane, and several peaks within the diesel oil chromatogram.

To monitor chemical changes in the oil during the degradation process, samples were removed from the oil layer at predetermined times, chromatographed, and analyzed by the peak height ratio method of Blumer et al (11) and a peak-envelope ratio method developed herein. Peak heights for the n-C16/Pristane, n-C17/Pristane, and n-C18/Phytane ratios were measured from the baseline to the top of the peaks. Peak heights for the n-C13/envelope and n-20/envelope ratios were measured from the top of the peaks to a line drawn across the bottom of the peak at the edge of the envelope. The envelope is defined as that area enclosed by the baseline and a line drawn along the bottom of all peaks in the profile. The term profile indicates the entire chromatographic pattern.

Figure 2 - Gas Chromatographic Profile of Standard Hydrocarbon Mixture Referred to as ARTOIL.



VI. RESULTS AND DISCUSSION

A. Characteristics of the Typical Oil Degradation Process in Continuous Culture - Physical Changes

The initial objective of our work was to establish a standard set of characteristics which would be typical of any degradation sequence observed in our continuous culture systems. Despite the time element involved, enough experiments have been performed to obtain a general description of how the oil is attacked. All conditions of our experiments were maintained as uniform as possible and all systems were incubated at room temperature until all of the oil had disappeared from the culture vessel. At present, we recognized five important phases in the degradation scheme and these are described below. They are also summarized in Table 1.

1. Adjustment Phase

This first phase constitutes the adjustment taken by the mixed bacterial population in the Lake Ontario inoculum in response to the presence of the oil. It involves a selection for those bacteria which associate rapidly with the oil layer. Since the oil has been topped and since many of the more soluble hydrocarbons are volatile, it is unlikely that the oil contributes much soluble organic matter to the growth medium. Therefore the bacteria which do not adhere to the oil have little substrate for growth and are unable to maintain themselves in the continuously diluted growth vessel. This reasoning is reflected in the initial observations of the oil degradation process. Characteristically a rapid increase in turbidity occurs within the first 2-5 days followed by a leveling off and eventually a slow decrease in the turbidity. Within about 100 (4 days) hours of incubation, the culture fluid became relatively clear and a significant lag period sets in. The immediate increase in turbidity was probably due to bacterial growth on small amounts of indigenous organic material present in the lake water inocula. This growth would have been stimulated by the high concentrations of nitrogen and phosphorous which were added at the time of inoculation. As the experiment progressed, however, this organic material was either used up or washed out of the culture vessel resulting in a decrease in the population density and causing the gradual decrease in turbidity.

The so called lag period is characterized by a general lack of change which is quite variable in length being of 4-10 days duration after inoculation in most experiments.

2. Surface Growth Phase

This second phase is characterized by the obvious increase of bacteria on the bottom surface of the oil layer. Typically a thin bacterial film developed at the oil water interphase causing the oil layer to become stagnant, i.e., it no longer moved in the current caused by the stirring bar. With

Table 1. Description of Physical Changes Occurring During a Typical Oil Degradation Process in Continuous Culture

Stage of Degradation	Point of Occurrance After inoculation (days)	Duration (days)	Description
Adjustment Phase	1-2	2-10	Rapid change over in predominant bacterial species Initial fluxuation in cell density Eventual clearing of culture fluid under oil layer General lag period - no gross physical change
Surface Growth Phase	4-6	4-25	Accumulation of bacterial film on bottom surface of oil layer Oil layer becomes stagnet Bacterial film thickens Wall growth
Impregnation Phase	10-30	10-until termination	Oil layer becomes turbid, milky brown in color Oil layer highly impregnated with bacteria Oil layer breaks up into mass of floating droplets Culture fluid relatively clear
Flaking-Off Phase	25-35	25-until termination	Masses of cells and oil flake off oil layer Oil layer disappearing Oil layer could be easily dispersed by increased turbulance at any time
Washout-out Phase	40-50	40-until termination	Most of oil layer gone Most of the bacteria washed out Some wall growth remains

further incubation, 170 to 600 hours (7-25 days) the bacterial film thickened and cell masses began to accumulate in and around the oil layer. Wall growth was also noticeable around the oil layer and on the center tube. Small air bubbles from the aeration process generally begin to accumulate under the oil layer. Again, the length this phase was variable, generally lasting 4-25 days.

3. Impregnation Phase

As the bacterial film continued to thicken, the oil layer itself became turbid due to the impregnation of the oil by the bacteria. The oil layer became milky brown in color and almost completely amassed in the thick mat of bacterial growth. Wall growth around the oil layer also increased significantly but the culture fluid itself was only slightly turbid with small quantities of particulate material interspersed throughout.

Also quite noticeable at this time was the beading up of the bacteria-impregnated oil layer into small droplets which accumulated around the center tube. We have termed this droplet formation as a form of emulsification since the droplets did not coalesce back into an oil layer. The drops probably formed as a result of agitation caused by escaping air bubbles and the entrance (drop wise) of fresh media. The persistence of the droplets was a function of the bacterial growth since similar agitation occurred in the control system without subsequent emulsification. As incubation continued, the emulsification increased until the entire oil layer was composed of small droplets amassed together.

4. Flaking-Off Phase

In this phase the amount of growth in the oil layer became so extensive that white particulate amorphous masses of cells and oil droplets began to flake off the oil layer. These were then mixed into the culture fluid, causing a slight increase in turbidity, and eventually washed out of the growth vessel. Microscopic examination revealed that these flakes consisted primarily of bacterial cells interdispersed with an emulsion of oil. The flaking process continually removed small amounts of the oil during the rest of the experiment until about 600 and 700 hours (25-29 days) when the flaking increased significantly giving the culture vessel a "snow fall" appearance. If at any time during the flaking phase, the revolutions per minute of the stirring bar was increased the amount of flaking increased and greatly speeded up the removal of oil from the oil layer. Under such increased turbulance, we have seen the oil disappear within two-to-three days. However, the object of our experiments was not to obtain maximal oil removal rates but instead to obtain a degradation process which we could study in detail over an extended period of time.

5. Washout Phase

After 700-800 hours (30 days) of incubation, the flaking started to decrease as the oil layer was now greatly reduced, it no longer covered the entire surface of the water column.

Finally, in 1060 to 1400 (50 days) hours, the oil layer completely disappeared, as did the turbidity in the culture. The wall growth also decreased but much slower.

These changes have been consistent from experiment to experiment with only variations in the time spans and intensity of each event. We have estimated that it takes from 1100 to 1400 hours incubation in our system to completely remove a 2 mm thick layer of oil from the surface of a water column under nonturbulent conditions.

Since bacteria appeared which can attack a stable oil-water interface, extensive dispersion is not a prerequisite for degradation. The method of attack does not involve the immediate use of emulsifying agents, since the oil remained in the continuous culture system for extended periods of time. Instead, the attack involved a direct attachment to the bottom of the oil layer, followed by a slow degradation and mild emulsification from the bottom upwards. The bacteria responsible for the initial attachment events appeared to be unique in that not every lake water sample contained them, (i.e. initiation of degradation of the oil was never observed and experiments were subsequently restarted). Many water samples taken during the summer months (May thru August) seemed particularly inadept at initiating the degradation of a stable oil-water interphase. We are not exactly sure what determines the success of this event but preliminary indications (see section E) are that one or two specialized types of bacteria are responsible and for some unexplained reason they simply may not be as plentiful in the Lake during the summer.

B. Characteristics of the Typical Oil Degradation Process in Continuous Culture - Chemical Changes in the Oil Layer

Bacterial growth and the disappearance of oil from the surface of a body of water does not, in itself, indicate degradation. The use of gas chromatography does allow one to obtain both a qualitative and quantitative estimation of the extent of degradation. In an attempt, therefore, to correlate the physical changes with the chemical changes brought about by the bacteria, gas chromatographic analysis was carried out.

1. Undegraded Oil

A gas chromatogram of the topped undegraded diesel oil used in our experiments is shown in Figure 3. Characterization of the chromatographic profile of the oil shows in order of prominance: 1.) a series of n-alkanes, n-C12 to n-25; 2.) two highly resolved iso-alkanes, pristane and phytane; and 3.) over fifty smaller peaks partially resolved from the envelope. The partially resolved peaks and unresolved envelopes are comprised of various iso-alkanes, cycloalkanes, and aromatic hydrocarbons.

A major problem which became apparent during the chemical analysis of the oil, was a loss of some of the lower boiling components during the concentration procedure, an effect which would invalidate some of the changes seen in the peak ratios. Data in Figure 4 and Table 2 show the results of an experiment to determine the effects of concentrating the sample. An

Figure 3 - Gas Chromatographic Profiles of Diesel Oil Taken from the Oil Layer of the Continuous Culture System X1 After Different Periods of Incubation: a) undegraded control, b) 700 hours, c) 1135 hrs, d) 1400 hrs.

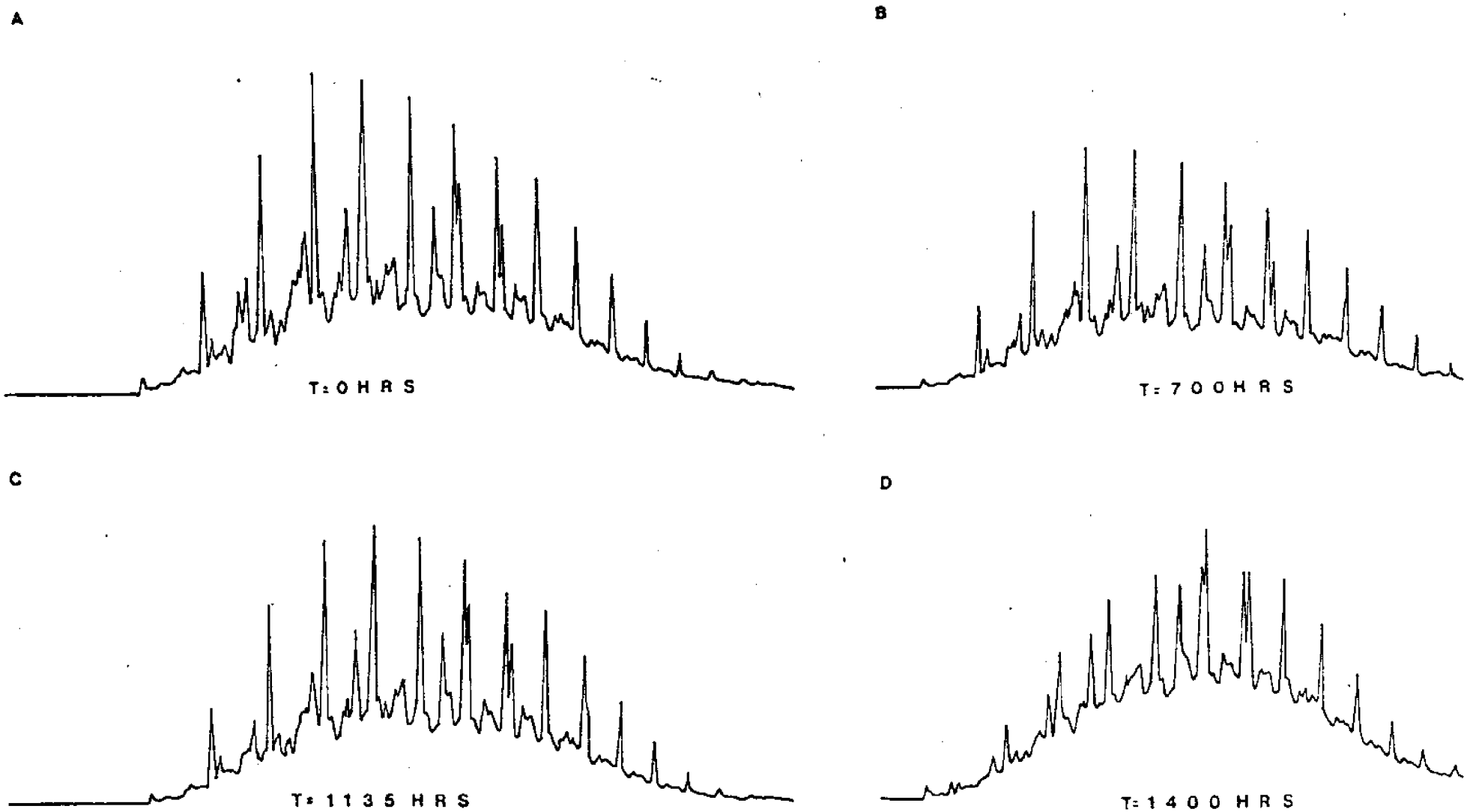
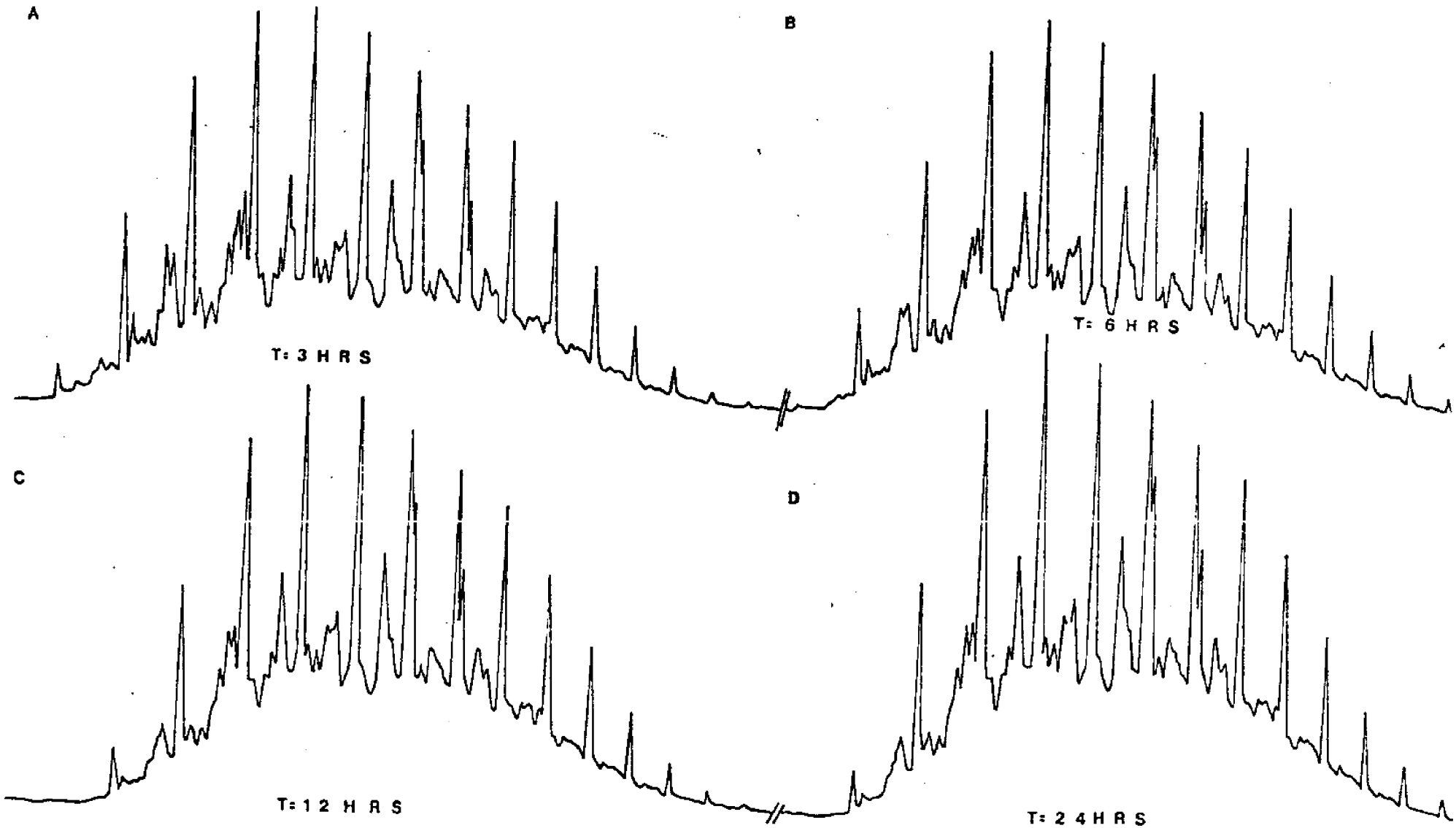


Figure 4 - Gas Chromatographic Profiles of Topped Diesel Oil Exposed to Different Periods of Forced Air Drying During Concentration Procedure.



examination of the profiles shows that some of the lower boiling compounds are indeed lost during drying down. But, even after 24 hours of forced draft conditions, the n-C17/pristane and n-C18/phytane ratios were only decreased to 1.18 (1.6% decrease) and 1.32 (4% decrease) respectively. Since no oil or effluent samples were ever exposed to the forced draft for more than 12 hours, these figures represent the minimum ratios due to the drying procedure. These changes were thus used as the limits of experimental error and any additional decrease beyond this was considered attributable to microbial activities. Data in Table 2 also shows the effects of drying on the n-C20/envelope and n-C13/envelope ratios. The latter ratio underwent the most drastic drop, from 2.16 to 1.85 (15% decrease). As will be shown later, this change in the ratios is much smaller than that observed in degraded oil. As might be expected, the n-C20/envelope ratio was not affected by drying down since n-C20 is a relatively non-volatile hydrocarbon.

2. Degraded Oil - 700 Hours Incubation

Figure 3 shows the chromatographic profiles of the undegraded oil as well as oil recovered from the oil layer of system XI at the noted times. Peak height ratio and peak-envelope ratio data, for these profiles are shown in Table 3.

In examining the data, it can be seen that there is very little change in the profiles as compared to the undegraded oil up to 700 hours of incubation. A slight change in the peak heights of the lower region can be seen, but these can probably be attributed to the concentration procedure noted above. Very little change was noticeable in the n-C13/envelope ratios above the effects of drying down.

3. Degraded Oil - 1135 Hours Incubation

Noticeable changes are apparent in the oil after 1135 hours of incubation (figure 3). The decrease in both the n-C17/pristane (11% decrease) and the n-C18/phytane ratio (16% decrease) indicates degradation of the n-alkane series especially the lower molecular weight n-alkanes. The n-C13/envelope ratio dropped significantly (40% decrease) when compared to both the undegraded oil and the n-C20/envelope ratio has dropped only slightly (7%). This is a strong indication that the small chain lengths are being preferentially attacked. The increased solubility of the lower boiling hydrocarbons and presumedly makes them more accessible to microorganisms (31, 34).

4. Degraded Oil - 1400 Hours Incubation

Even more pronounced changes are evident in the oil residue removed at the termination of the experiment (figures 1400 hr). All of the ratio data indicates a degradation of the n-alkane series. For example, the n-C17/pristane and n-C18/phytane ratios show a 26% and 25% decrease respectively when compared to the undegraded oil. This is substantiated by large decreases in the n-C20/envelope and n-C13/envelope ratios. Although, these changes are not readily apparent in the gas chromatographic profiles shown in figure 3 due to their reproduction (and reduction) for this report, first hand examination does show the indicated changes.

Table 2 - Peak Height Ratios from Gas Chromatographic Profiles of Topped Diesel Oil Exposed Differing Periods of Force Air Drying During Concentration Procedure.

Time Under Draft Conditions (Hours)	n-C17/Pristane Ratio	n-C18/Phytane Ratio	n-C20/Envelope Ratio	n-C13/Envelope Ratio
3.0	1.20	1.38	1.19	2.16
6.0	1.21	1.33	1.18	2.10
12.0	1.18	1.35	1.19	1.83
24.0	1.18 (2%)	1.32 (4%)	1.19 (1%)	1.83 (15%)

A slight alteration of envelope components may also have occurred, since, the profile has become slightly skewed to the right. This would be expected if some of the cyclic alkanes and aromatics were degraded. However, at this point we have no way of quantitating the change.

A similar set of data obtained from a second run of the same experiment (designated 2) is shown in Figure 5 and Table 3. This degradation run was under the same set of conditions but inoculated with a different Lake Ontario water sample. Except for some minor changes, the degradation follows the same pattern as in the X1 experiment.

5. Discussion

It is evident from the above data that the degradation of the oil in the oil layer is a very slow process. Even after 1300 hours of incubation, System X1 and System X2 showed only a small degree of degradation.

This is a difficult situation to explain since the oil was in fact removed from the oil layer (through the flaking process) and massive amounts of growth occurred in and around the oil layer. It is possible that because of the thickness of the oil layer, degraded oil was diluted to the point of obscurity by undegraded oil. This dilution effect would also account for the progressive increase in the amount of degradation seen with longer and longer periods of incubation. It is also possible that degraded oil occurred predominantly at the oil-water interface and thereby escaped detection as it was leached into the water column below the oil layer. We therefore examined the water passing under the oil layer for the presence of degraded oil.

C. Characteristics of the Typical Oil Degradation Process in Continuous Culture - Chemical Changes in Effluent Oil

In an attempt to account for the lack of microbially mediated chemical alteration of the oil in the oil layer despite the massive amounts of growth obtained, we decided to examine the oil in the effluents (i.e., the water passing under the oil layer and out of the culture vessel) from our continuous culture systems.

It was also clear to us at this point that if the flaking off phase occurred under natural conditions, and there is no reason to believe that it would not, the major part of any spilled oil would be dispersed in the water column rather rapidly (i.e. disappear from sight) in a relatively undegraded form (as we have observed from our results). The advantage of our continuous culture system is that it mimics this dispersion process but in a slow controlled manner. Thus, it becomes feasible to chemically analyze the oil as it is dispersed (i.e. as it comes off in the effluents of the growth vessels).

1. Effluent Oil - 169-333 Hours Incubation

Effluents from the continuous culture systems were removed and extracted as outlined in Materials and Methods.

Table 3 - Peak Height Ratios from Gas Chromatographic Profiles of Diesel Oil Taken from the Oil Layer of the Continuous Culture System X-I and X2 After Different Period of Incubation.

	Incubation Time (hrs)	n-C17/Pristane Ratio	n-C18/Phytane Ratio	n-C20/Envelope Ratio	n-C13/Envelope Ratio
System X1	0	1.21	1.34	1.30	2.16
	700	1.22 (1%)	1.35 (1%)	1.26 (3%)	1.84 (15%)
	1135	1.08 (11%)	1.12 (16%)	1.20 (7%)	1.22 (44%)
	1400	0.89 (26%)	1.00 (25%)	0.80 (38%)	0.68 (69%)
System X2	0	1.21	1.37	1.30	2.16
	627	1.22 (1%)	1.38 (1%)	1.16 (11%)	2.17 (1%)
	1177	1.10 (9%)	1.26 (8%)	1.04 (20%)	1.41 (35%)
	1323	1.03 (14.8%)	1.17 (14.5%)	0.92 (29%)	1.16 (46%)

The gas chromatographic profile of the oil removed at 169 hours incubation, and the hydrocarbon peak ratio data are shown in Figure 6 and Table 4. There is a much more extensive degradation of the oil recovered from the effluents than oil recovered from the oil layer. Most obvious is a general decrease in almost all of the n-alkane series especially those below 17 carbon atoms. The n-20 alkane/envelope ratio appears to show the greatest change (30-40%) in comparison with the pristane and phytane ratios. However, if one calculates the change in the n-17 alkane/envelope ratio (28%) and the n-18 alkane/envelope ratio (28%) they do not significantly differ from the change in the n-20 alkane/envelope ratios. For example, at 300 hours, the n-17 and n-18 alkane ratios each changed approximately 28% which compares closely with the 31% change observed for the n-20 alkane/envelope ratio. Thus, in fact, all the saturates appear to be degraded at the same rate or in the same manner.

The large difference seen between the changes in the pristane-phytane ratios and the changes in the n-20 alkane/envelope ratios, indicates that both pristane and phytane are being degraded. Presumedly, if pristane and phytane are as recalcitrant as the envelope components, all of the above ratio changes should be approximately the same, assuming there is no preferential degradation within the alkane series. This was not the case, and in fact, pristane and phytane envelope ratios did change by approximately 20%, thus indicating that they were being degraded.

It is also clear, however, that envelope components are being altered as well. As can be seen in Figure 6 at 169 hours incubation, the envelope profile (that is, an imaginary line drawn through the base of all peaks) significantly differs from the envelope profile of the undegraded oil (superimposed dotted line). Typical of this change is a loss of lower boiling components (left side of profile) and an increase in the higher boiling components (right side of profile). Note, too, that the point of maximum concentration of envelope hydrocarbons has shifted to the right. It would seem that this at least represents degradation of the lower molecular weight aromatics and cyclic alkanes and perhaps even a synthesis of higher molecular weight hydrocarbons. This latter prospect has already been documented in the literature (50).

Further examination of the profiles reveals an apparent enrichment for several unknown peaks in the beginning and the end of the profile. This enrichment could result from compounds which are resistant to microbial degradation and therefore become a larger proportion of the profile as the other hydrocarbons decrease. There have not been identified as yet although retention data indicates that the first large peak could be a polyaromatic hydrocarbon such as 1-methylnapthalene. Napthalenes, on the other hand, are not known to be particularly recalcitrant, i.e. it is relatively easy to detect organisms which can degrade them. It is possible, however, they could be more resistant to attack as a result of the degradation process occurring in the continuous culture system. A diauxic relationship, for example, with the alkanes may exist. It is also possible that napthalene utilizers in the system were washed out. To date, we have no evidence to indicate that they are contaminants from our gas chromatographic column. Their validity and identification will have to wait for further experimentation.

Figure 5 - Gas Chromatographic Profiles of Diesel Oil Taken from the Oil Layer of the Continuous Culture System X2 After Different Period of Incubation: a) Undegraded Control; b) 627 hrs; c) 1179 hrs; d) 1323 hrs.

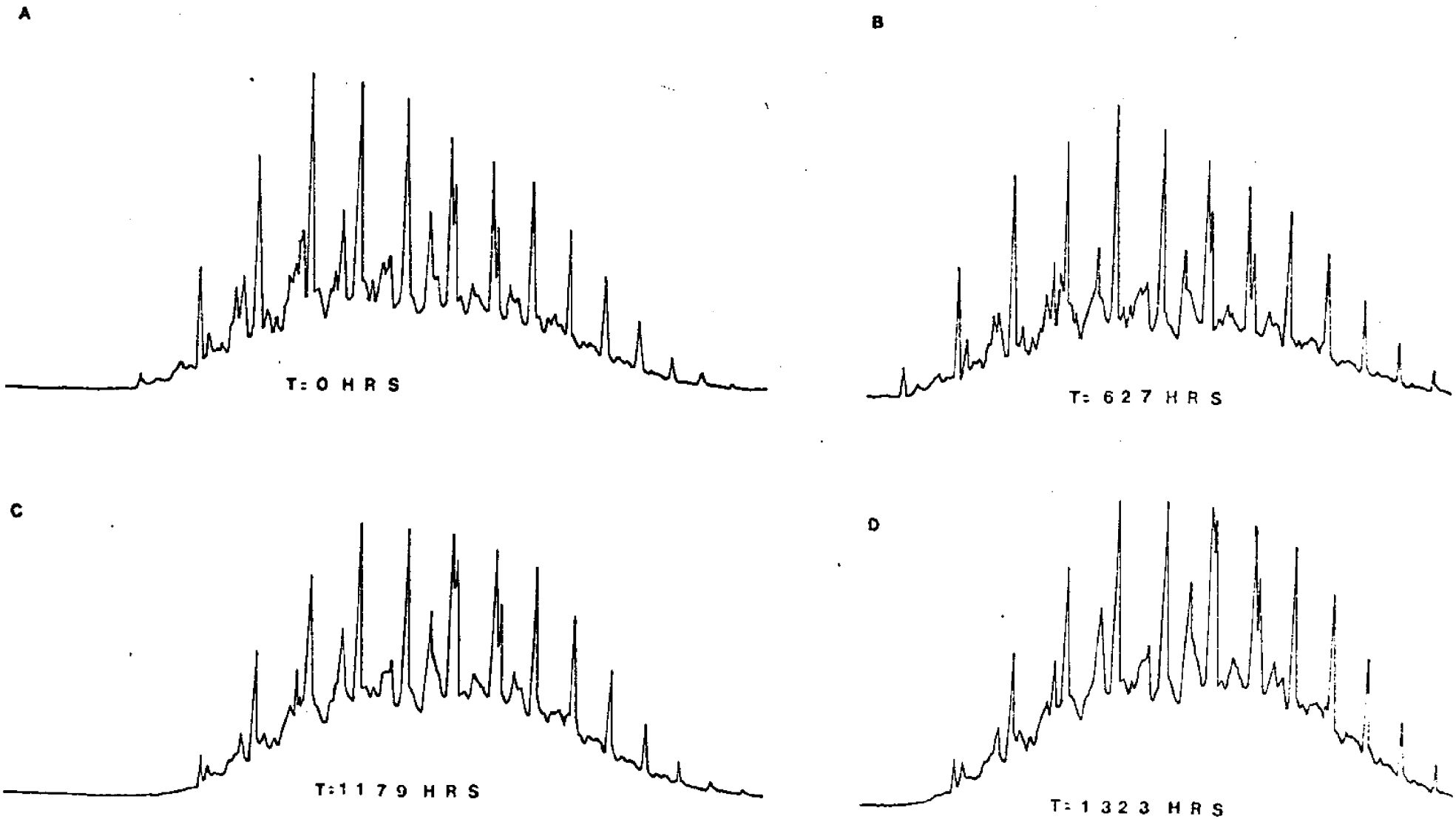


Figure 6 - Gas Chromatographic Profiles of Oil Extracted From the Effluents of Continuous Culture Systems After Different Periods of Incubation.

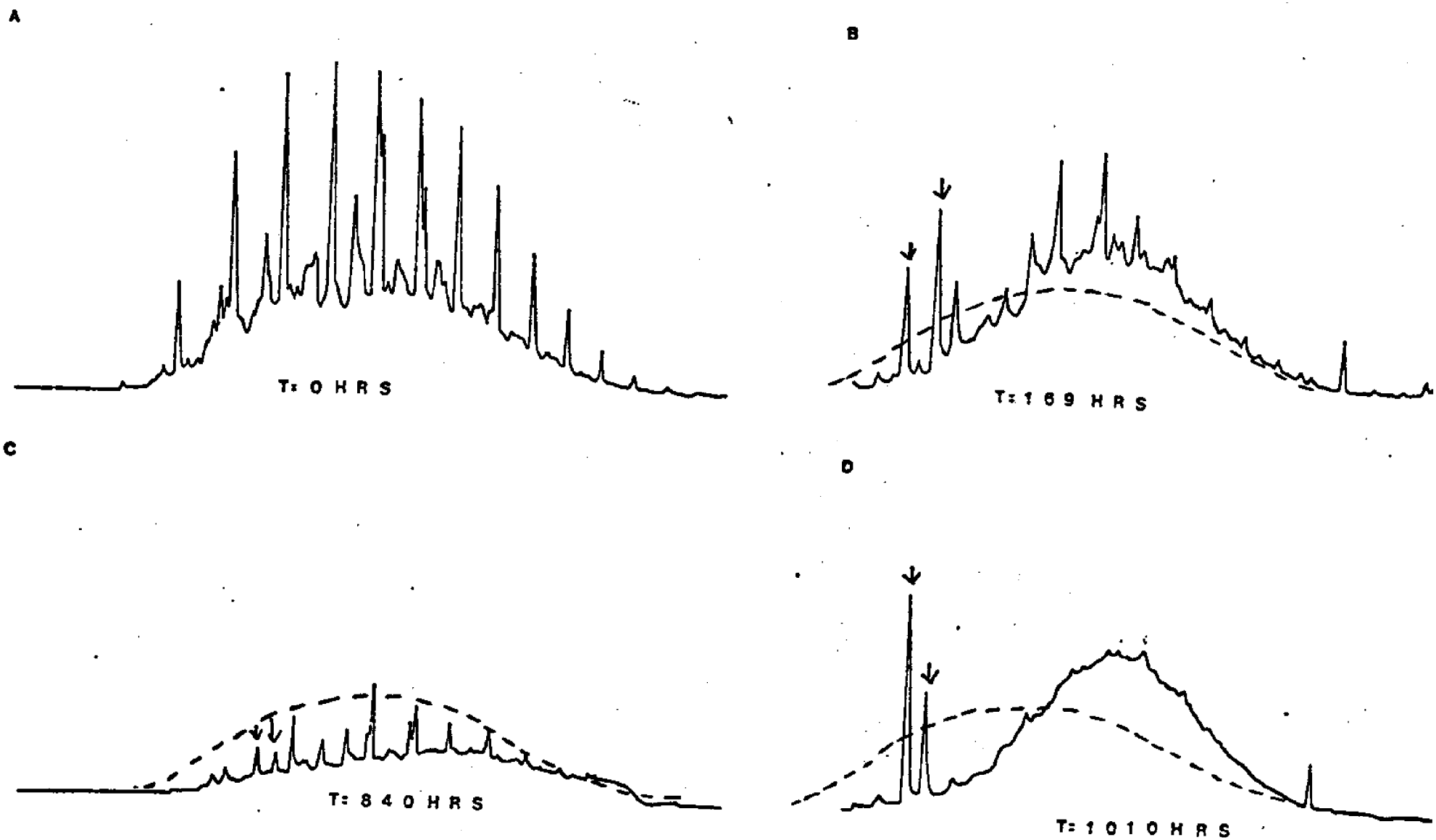


Table 4 - Peak Height Ratios From Gas Chromatographic Profiles of Oil Extracted From the Effluents of the Continuous Culture Systems After Different Period of Incubation.

Incubation Time (hrs)	n-C17/pristane ratios	n-C18/phytane ratios	n-C20/envelop ratios
0	1.21	1.34	2.90
169	1.12 (7.5%)	1.25 (6.2%)	2.55 (12.2%)
333	1.04 (14.0%)	1.18 (11.8%)	2.0 (31.0%)
500	0.76 (37.5%)	1.02 (23.8%)	1.55 (46.6%)
673	0.72 (40.0%)	0.75 (44.0%)	1.19 (59.0%)
840	0.58 (52.0%)	0.81	1.41
1010	>> 1*	0.76	1.24
1323	0.735	0.84	1.14

*Peak height too small to measure.

2. Effluent Oil - 500-840 Hours Incubation

During this period of incubation the degradation of the effluent oil continued to increase (figure 6). The alkane series was further reduced, including pristane and phytane but to a lesser extent, and the changes in the envelope profile represented a further accentuation of earlier observations. The apparent smallness of the chromatographic profile was the result of the sample volume injected into the gas chromatograph and not a function of the degradation process.

The large enriched peaks that were seen in the 169 hour profile are also present in this profile but they are not as prominant (see arrows in Figure 6). This could be the result of further degradation. However, as can be seen in the 1010 hour sample, these peaks again return to their original prominance. It is difficult at this time to explain the concentration variation of these unknown peaks.

3. Effluent Oil - 1010-1323 Hours Incubation

At this point in our experiment the alkane series and the more recalcitrant branched alkanes were extensively degraded and the envelope profile had been extensively modified. However, if the ratios are examined in Table 4 (these ratio are only estimates due to the smallness of the remaining peaks) it is apparent that they have become variable and do not necessarily go to zero. This is probably both a function of gas chromatographic resolution and the possible input of small amounts of undegraded or partially degraded oil flaking off the oil layer. It may also reflect a steady state rate of degradation.

4. Discussion

It is clear, from our experiments, that the oil in the oil layer was degraded at a very slow rate. This is in contrast to many laboratory studies using batch culture systems in which a more rapid degradation is observed (29). These systems, however, involve a much more vigorous type of agitation making the hydrocarbons more excessible to the cells. Likewise, the population densities typical of batch culture may also unnaturally increase the degradation rate.

It is quite significant for our results that Blumer et al (10) have observed essentially the same degradation with diesel oil in the marine environment. For example, they showed that the n-C17 to pristane ratio decreased from 2.0 to 1.5 (25% change) within 2 months (1400 hours). Ratios obtained in our systems are comparable to this data. Blumer obtained his samples directly from the sediments and we would envision this as being equivalent to taking samples directly from our oil layer. Thus our system appears to provide a realistic laboratory model for the study of the environmental persistance of different hydrocarbon types in the oil.

Many, if not most, oil degradation studies done in batch culture also show primarily a removal of the predominant n-alkanes leaving the envelope essentially intact. Only after considerable lengths of incubation do they

ever show alteration of the envelope components and then only to a slight degree. Our work indicates a significant attack on both the n-alkanes and the envelope hydrocarbons.

Effluent samples have shown that the oil was degraded to a much greater extent than in the culture fluid (Figure 4). This is an important factor which has not been considered in analysis of oil in the environment. If only the residual spilled oil is sampled for degradation it may appear, as it did in our study, that the oil is not being significantly degraded. However, if the water column around the residual spilled oil is sampled (equivalent to sampling the effluents in our continuous culture systems) extensive degradation would probably be detected. It is necessary then, to sample the water surrounding an oil slick as well as the oil itself in order to determine the extent of degradation. Blumer's work (10) again emphasizes this point: he detected degradation or alteration of the envelope components of diesel oil only after extended exposure (3 months to a year) to the microbial activities in the sediments. This was accompanied with a general spreading of the oil over a large surface area of the sediment interfase. This further points out the apparent critical need for movement of the oil through the water column or any other type of milieu in order to get extended degradation (namely the cycloalkanes and aromatics) of the oil. Possibly if Blumer had been able to sample water above the oil contaminated sediments he would have detected a degradation pattern similar to what we have seen in our effluents under the same time period. This lack of movement of the oil and its relationship to degradation is further exemplified in the work of Jobson et al (29) who have shown a completely intact envelope profile of crude oil immobolized in a soil plot for 308 days.

Another interesting aspect of these results is the fact that oil taken from the effluent samples shows greater and greater degrees of degradation as the incubation period increases. If our systems were operating as quasi continuous culture system, one would not expect this result; instead it would be expected that small quantities of oil would be slowly leached off the oil layer and that, as they passed thru the culture fluid and eventually washed out of the vessel, they would be exposed to the same amount of degradation (steady state). This, as was seen, was not the case except perhaps during the latter stages of the experiment where ratio changes began to level off. There are two possible explanations for this lack of a constant degradation pattern. First, it may be that the bacterial population composition was continuously changing and that as the experiment progressed, it became more and more efficient at degrading the oil coming out in the effluents. Second, as the number of bacteria increased in the oil layer, more and more alteration of the oil would have resulted. This would mean a more degraded oil entering the culture fluid and would enhance its subsequent microbial degradation in the culture fluid before it actually washed out in the effluent. The process is undoubtedly more complex than this but it will have to wait further experimentation.

D. Changes in Bacterial Populations During Oil Degradation

Because of the extensive physical and chemical changes in the oil which are brought about by the bacterial attack, it became of interest to us to see if we could determine the types of bacteria involved and the existent interactions which allowed them to degrade the oil. To do this, samples of culture fluid from the continuous culture systems were simply assayed for the total numbers and types of bacteria present during the degradation process.

1. Enrichment and Predominant Species

The total bacterial population, which was very heterogeneous at the time of inoculation, changed rather rapidly to a population composed of 4 or 5 types which accounted for about 85-95% of the total. Throughout most of the experiments only four or five types were present as predominant species (see Table 5).

The predominant species found in the culture fluid were generally the same as those found associated with the oil. Substrate specificity tests also indicated that there was no significant difference in predominant species from the two sampling areas.

Population densities in the culture fluid were surprisingly invarient, maintaining densities for 10^5 - 10^6 cells/ml. Considering the tremendous complexity and insolubility of the substrate employed, a mixed population approaching steady state growth would not be expected.

The four or five predominant species present during most of the degradation did not remain the same. There was a gradual change from small, white smooth colonies, to slightly larger yellow pigmented colonies, to eventually very large and slimy colonies. This changeover in colony types is very apparent when viewed all together and we are now attempting to correlate these changes with changes in substrate specificity and changes in the chemical composition of the oil.

2. Substrate Specificities of Bacterial Species from Continuous Culture Systems

Theoretically, the occurrence of a predominant species, or the change over to a different predominant species could indicate that conditions had changed in the growth vessel allowing a successful competing species to outgrow all other species and eventually predominant. The particular set of conditions which set up this situation could result from a simple change in the chemical composition of the oil brought on by the degradation process. Thus one would expect that the substrate specificity of a particular predominant species would be complementary to the change which had occurred in the oil.

Substrate specificities tests were therefore conducted by isolating and purifying certain predominant bacterial types (as determined by colony morphology) and then heavily inoculating them into minimal salts medium

Table 5 - Predominant Types of Bacteria Present in Culture Fluid During Degradation of Oil in Continuous Culture Systems X1 and X2. Numbers are Expressed as Percent of Total Plate Counts.

Incubation Time (hrs.)	System X1	System X2
240	40% 2-3 mm white, smooth, raised	60% 4-5 mm yellow, spreading, flat
	20% 2-3 mm orange-yellow smooth, flat	20% 2-3 mm light yellow, depressed center
	20% 3-5 mm white, rough-edged	20% 1mm white, raised
480	40% 6mm tan, concentric pattern	60% 3-4 mm gray, raised center
	20% 3-4 mm dark orange, smooth	10% 5-6 mm tan, spreading, flat, dense center
	20% 4-5 mm white, slimy, globular	15% 4-5 mm lime-white, raised, rough edged
720	40% 4-5 mm white, slimy, globular	75% 4-5 mm lime-white, raised, rough edged
	20% 6mm dark tan, white smooth halo	20% 2-3 mm yellow, flat, dense center
	20% 3 mm lemon yellow smooth	
960	40% 1 mm white, opaque, smooth	40% 1 mm very white, flat, translucent
	20% 4-5 mm white, slimy, finger-like edge	20% 4-5 mm lime-white raised, rough edged
	20% 2-3 mm creamy light yellow, smooth	20% 1 mm white, raised, dry
	15% 6 mm dark tan, white edged	
1200	40% 4-5 mm white, slimy, finger-like edge	40% 4-5 mm lime-white, raised, rough edged
	20% 2-3 mm creamy light yellow, smooth	20% 1 mm very white, flat, translucent
	20% 6 mm dark tan, white edged	20% 1 mm white, raised, dry

Table 6. Substrate Specificities of Predominant Bacterial Species Isolated During the Degradation of Diesel Oil in Continuous Culture

	Substrate										
Isolate	C-8	C-10	C-16	C-20	Pris	TMB	nBB	Dec	Nap	Anth	Ac
A1	0	0	+	0	+	0	0	0	0	0	+
A3	0	0	+	+	0	0	0	0	0	0	+
A9-2	0	0	+	0	0	0	0	0	0	0	+
AE-18	0	0	0	0	0	0	0	0	+	+	+
AG-19	0	+	0	0	0	0	+	0	0	0	+
AM-30	0	0	+	0	0	0	0	0	0	0	+
Rx711	0	0	0	0	0	0	0	0	+	0	+
xx726	0	0	0	0	0	0	+	0	0	0	+
Sx711	0	0	+	+	+	0	0	0	0	0	+
Dx625	0	0	+	+	+	0	0	0	+	0	+
Ex725	0	slight	+	+	0	0	0	0	+	0	+
Lx78	0	0	0	0	0	0	0	0	0	0	+
AX10	0	0	0	0	0	+	0	0	0	0	+

C-8 = octane

C-10 = decane

C-16 = hexadecane

C-20 = eicosane

Pris = pristane

TMB = trimethyl benzene

nBB = n-buty benzene

Nap = naphthlene

Anth = anthracene

Ac = acetate

each containing 0.1% of one of a variety of representative hydrocarbons. Positive growth was deduced from increases in turbidity. A typical substrate specificity experiment for several predominant bacteria from the same experiment is shown in Table 6. Unfortunately, these experiments are very time consuming and not yet totally reliable. To date, we have not been able to detect any sort of correlation between the metabolic capabilities of our isolates and chemical or physical changes in the oil. However, hydrocarbon utilizers were definitely present. They can be divided into three types; those that use alkanes only, those that use only aromatic and those which grow on both alkanes and various aromatic hydrocarbons. Octane, hexadecane, and the fatty acids were the substrates most frequently attacked by our isolates. Anthracene and 1, 2, 4-trimethyl benzene were only rarely attacked, if at all. Also present, were many predominant species which did not attack hydrocarbon but readily grew on the other non-hydrocarbon substrates. Their significance in the oil degradation process is not yet clear but future experiments involving oil degradation by mixtures of pure culture help elucidate their role.

3. Discussion

This course of events, involving a series of enrichments is a well known phenomenon of continuous culture studies (52, 28). Jannasch (28) for example using simple sugars and organic acids as substrates, has shown an enrichment of one metabolic and morphological type from a heterogeneous population. The explanation for this phenomenon is that organisms which are growing slower, or not at all, are diluted more rapidly than the population of the faster growing species, yielding an enrichment of the latter. Theoretically, as seen in Jannasch's work the most successful utilizer should approach a pure culture state.

In the case of a complex substrate, such as diesel oil, however, one might expect to see multiple enrichments, assuming that fresh heterogeneous bacterial populations are supplied to the system continuously (as in the natural aquatic environment). The factors which then determine these enrichments are, the availability of the different hydrocarbon components, the presence of bacterial degradation products, and the restraints imposed by the surrounding environmental conditions.

In our continuous culture systems, fresh bacterial populations are not continuously supplied (i.e. the only source of bacteria is the original inoculum) and yet multiple enrichments are still observed. Thus, it appeared, that the bacteria which originally attached to the oil layer possessed the metabolic potential as a population to eventually remove all of the oil from the water's surface. It would be expected that if normal alkanes were preferentially attacked by the bacteria in the continuous culture systems, they would eventually out compete the aromatic hydrocarbon utilizers and the latter group would wash out of the continuous culture system. When the remaining oil then became enriched with aromatic hydrocarbons (due to the initial preferential degradation of the alkanes) the aromatic utilizers would be absent and degradation would slow down and probably stop. In the majority of the experiments run so far this has not been the typical sequence of events; instead all of the oil is eventually removed or degraded. Therefore, either the oil is removed by some physical process (involving only a small amount of degradation) or the bacteria are degrading the oil indiscriminantly and therefore preventing the wash out of important metabolic types.

It is reasonable to also assume, that since a large mass of cells always remained associated with the oil layer, none of the bacteria in the original inoculum ever really washed out; instead they remained attached to the oil as minor members of the population and therefore undetectable by our plating techniques. If this was the case, however, one would expect the reappearance of certain species of bacteria but this has not occurred in any system so far tested. Thus one is left with the conclusion that the removal of the oil from the oil layer is not a continuous uniform stripping of identical samples, but instead represents a continually changing, heterogeneous attack on the oil and its component hydrocarbon. Both the population data and the gas chromatographic data reflect this process. If this conclusion is correct, then an analysis of the metabolic potential of each predominant species should reveal a pattern indicative of the particular fraction oil being attacked by that time. We have been unable to date to generate such a correlation.

E. Microbial Seeding Experiments

Since large numbers of hydrocarbon degrading bacteria, with extensive metabolic capabilities can be readily isolated from different environments, it has been suggested that their degradative potential could be exploited for purposes of controlling oil spills. The primary prerequisites for such a suggestion involve the ability to develop an oil degrading mixed population of bacteria which can be readily accessible in a viable state at any time and a mechanism of application of the seed culture to assure proper degradation and removal of the oil. Commercial "oil-eating" cultures are presently available but several lines of evidence (32, 7), including our own, indicate that the oil degradation process may be too complicated to mimic with seed cultures. To further substantiate this claim, we tried several types of seeding procedures with our continuous culture systems.

1. Reconstitution Experiments With Pure Cultures

Bacterial cultures which represented the most predominant types present during an entire degradation experiment were isolated, cultured as pure cultures, and the mixed together. This mixture was used to inoculate a new sterile continuous culture system to see if the same degradation pattern could be observed. In all cases this mixture did not attack the oil and washed out of the growth vessel. We have been unable to obtain any degradation regardless of the combination of isolates employed even though we know these isolates will attack hydrocarbons and oil in batch culture. Thus, it appeared that a specific bacterial type or types which we have not been able to isolate, is required for some type of initial process which allows these cells to begin attacking the oil in its nondispersed state.

2. Reinoculation Experiments With Effluent Cultures

Several hundred milliliters of effluent from a continuous culture system covering the first 3-4 days incubation after inoculation were collected and refrigerated. This effluent culture was then used to reinoculate the same continuous culture system on a continuous tri-weekly basis. In most cases this type of procedure had no enhancing effect on the degradation; it simply

proceeded as normal through the various phases described earlier. We surmised that once the degradation process has been initiated, further enrichment with the initiating culture does not speed up the process, except perhaps under conditions where the oil is mechanically dispersed.

In several experiments, however, reinoculation with the effluent culture brought on a rapid emulsification process causing the oil to be washed out of the growth vessel within 4-8 days. Examination of the oil in the effluent showed only slight degradation of the alkanes. Thus despite the fact that the oil can be made to rapidly disappear (one objective in oil seeding procedures) it is entering the water column in undegraded form. These latter experiments have been difficult to repeat on a routine basis.

3. Inoculation With Oil-Degrading Batch Cultures

Similar results have also been obtained by inoculating fresh sterile continuous culture systems with batch culture enrichments. Enrichments obtained by shaking oil with a fresh Lake Ontario water sample supplemented with minimal salts for 10-days or until significant turbidity appeared, were fully able to initiate the normal degradation process in continuous culture. On rare occasions the batch culture enrichment induced premature emulsification resulting in rapid oil removal but very little degradation.

On the other hand, as soon as a batch culture enrichment on oil is serially transferred several times to sterile minimal salts media containing fresh oil, the resulting enrichment becomes unable to initiate the degradation process in continuous culture and in fact readily washes out of the growth vessel. Although these experiments are only in their preliminary stage, it tentatively appears that the procurement of seed cultures from batch culture enrichments may not necessarily produce efficient oil degrading populations.

4. Inoculation With Commercially Available Seed Cultures

Gerald C. Bower and Inc. produces an oil eating degrading mixed culture of spore formers called DBC Plus, type R-5. Our experiments with these cultures have shown that they do partially degrade oil in batch culture but they are totally ineffective in our continuous culture systems (i.e. they rapidly wash out). These prepared cultures would appear to possess good emulsification properties but poor degradation capabilities when tested in batch culture experiments.

F. Effects of Supplements on the Degradation of Diesel Oil

One important aspect of oil degradation which has not been studied to a great extent is the effect of the presence of readily degradable organic material on the oil degradation process. In sewage polluted waters, one would expect to find many types of organic material which would influence the population of bacteria present. The addition of straw or hay to an oil slick for clean-up procedures also adds a large quantity of organic material.

The presence of high concentrations of other organic material may inhibit the degradation process. Catabolite repression, or the inhibition of the utilization of one metabolizable compound in the presence of the other, is a well known physiologically characteristic of microbial growth of pure cultures but it may also affect mixed cultures in natural situations. If, for example, a population is growing on a non-hydrocarbon substrate it may not be able to metabolize the oil at all, whereas if the oil was the sole carbon and energy source it would be readily utilized. In fact, the presence of fatty acids has been shown to cause inhibition of growth on a crude oil (5).

On the other hand, degradation may also be increased. For example, the presence of certain organic compounds may increase the microbial degradation by co-oxidation (27, 46).

Additional carbon sources may also cause preferential utilization of certain fractions of the oil. The addition of n-hexadecane for instance, could increase the utilization of the n-alkane series by causing an enrichment of the population specializing in their degradation. The same may be true for aromatic and cycloalkanes hydrocarbons.

Since we have now established a typical pattern of oil degradation by bacteria in our continuous culture system it was possible to investigate the effects of various organic supplements on the degradation scheme.

1. Glucose Supplementation

The addition of 0.1% glucose to the reservoir media had a profound effect on the degradation process. The oil layer did not undergo any of the physical changes noted in the X experiments, nor did bacteria associate with the oil layer and the oil layer remained unemulsified through out the entire experiment. The media however became extremely turbid with growth within 240 hours and remained in this state throughout the experiment.

When oil was removed from the oil layer at the termination of the experiment (t=764 hrs.) and subjected to gas chromatography it was obvious that the total profile remained almost identical to the stock oil (Fig. 7). Peak height ratio data (Table 7) substantiates this lack of change. Both the n-C17/Pristane and n-C18/Phytane ratios remain essentially identical to the control oil. It is apparent then that the addition of glucose to the salts media significantly decreases the degradation process seen in the unsupplemented X experiments. No oil was detected in the effluents.

Likewise, there are distinct differences in the development of the bacterial population in the presence of glucose. There was an enrichment for only two types of bacteria as early as 120 hours incubation and the two organisms eventually constituted 95% of the population. The only change which occurred after the enrichment was a change in predominance between the two organisms. Early in the experiment, species 39, a white, raised colony with rough edges constituted 75% of the total cell count while species 40, a white, raised, smooth colony was approximately 20%. This slowly changed until species 40 became 70% while species 38 dropped to 35% predominance.

Figure 7 - Gas Chromatographic Profiles of Diesel Oil Taken From the Oil Layer of Continuous Culture Systems Containing 0.1% Glucose (b) or 0.1% Propionate (c) in the Reservoir.

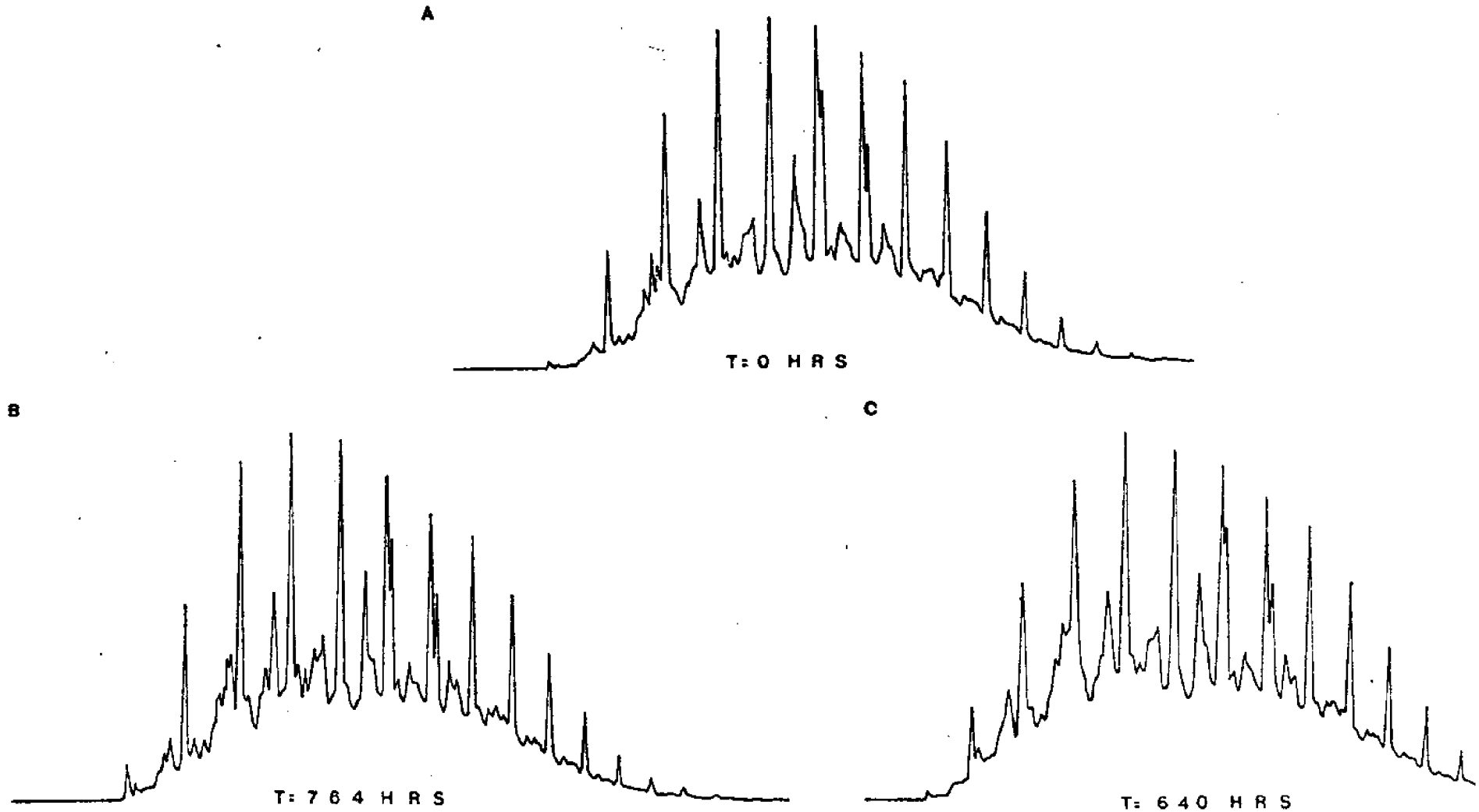


Table 7 - Peak Height Ratios From Gas Chromatographic Profiles of Oil Taken From Oil Layer of Continuous Culture Systems Containing 0.1% Glucose or 0.1% Propionate in the Reservoir.

System	Incubation Time (hrs)	n-C17/Pristane Ratio	n-C18/Phytane Ratio
Stock Oil	0	1.21	1.37
Glucose	764	1.21 (0%)	1.35 (1%)
Propionate	640	1.20 (0.8%)	1.35 (1%)

As in the unsupplemented systems, the inoculum contained about 10^4 cells per milliliter but this increased to 10^7 cells per milliliter and remained at this level during the course of the experiment. This was an order of magnitude higher than the population density seen in the unsupplemented system. The cell count probably represents only a conservative estimate of the population present, since there was large amounts of wall growth in the culture vessel.

This enrichment process again agrees well with previous work on continuous culture enrichment (28). It is evident that this enrichment for the two glucose utilizing organisms (which could not grow on oil or hydrocarbons in batch culture) was responsible for the inhibition of the typical degrading process. Presumedly, other organisms capable of attacking the oil could not establish themselves in the culture vessel and were washed out.

2. Propionate Supplementation

When sodium propionate was added to the resevoir at a final concentration of 0.1% again no degradation occurred (table 1, figure 7). Visual examination of the oil showed a complete lack of bacteria associating with and emulsifying the oil. As with the glucose system there was no chemical change in the oil and the n-C17/Pristane and nC18/Phytane ratios remained essentially constant.

The initial heterogeneous population of the inoculum increased only slightly from 10^4 to 10^5 cells per milliliter and an enrichment for two species occurred as early as 480 hours incubation (24 retention volumes). Over 95% of the population consisted of a large yellow-tan raised colony and a smaller white raised colony throughout almost the entire experiment. Each species constituted approximately 50% of the total plate counts.

3. Hexadecane Supplementation

The addition of 1 milliliter hexadecane to the oil layer (7 ml) had little, if any, effect typical oil degradation process seen in continuous culture. The media became cloudy within the first 100 hours after which bacterial growth formed under the oil and emulsification was initiated. The oil layer became totally emulsified by 600 hours incubation and began to clump and flake off. At the termination of the experiments (between 765 to 987 hours) a greater volume of oil remained in the oil layer than was seen in the unsupplemented systems at the same time interval.

Gas chromatographic profiles of oil from the oil layer (Figure 8) and the resulting peak ratio (Table 8) indicate a degradation pattern similar, although slightly more extensive, than that obtained during a normal unsupplemented oil degradation process at the same incubation time. Systems H1 and H2 are identical experiments but starting with different inocula. No significant change in the n-C17/Pristane and n-18/Phytane ratios was noted for either systems H1 or H2. Peak envelope data does show some degradation of both n-C13 and n-C20. The decrease of these alkanes, at this time in the experiment was not seen in the unsupplemented X systems at a similar, thus showing that degradation was increased to a small extent in the supplemented system.

Figure 8 - Gas Chromatographic Profiles of Diesel Oil Taken From the Oil Layer of a Continuous Culture System Containing Hexadecane in the Oil Layer (1:6 v/v Hexadecane: Oil) After Different Period of Incubation. Systems H1 and H2 Represent the Same Experiment With Different Starting Inocula.

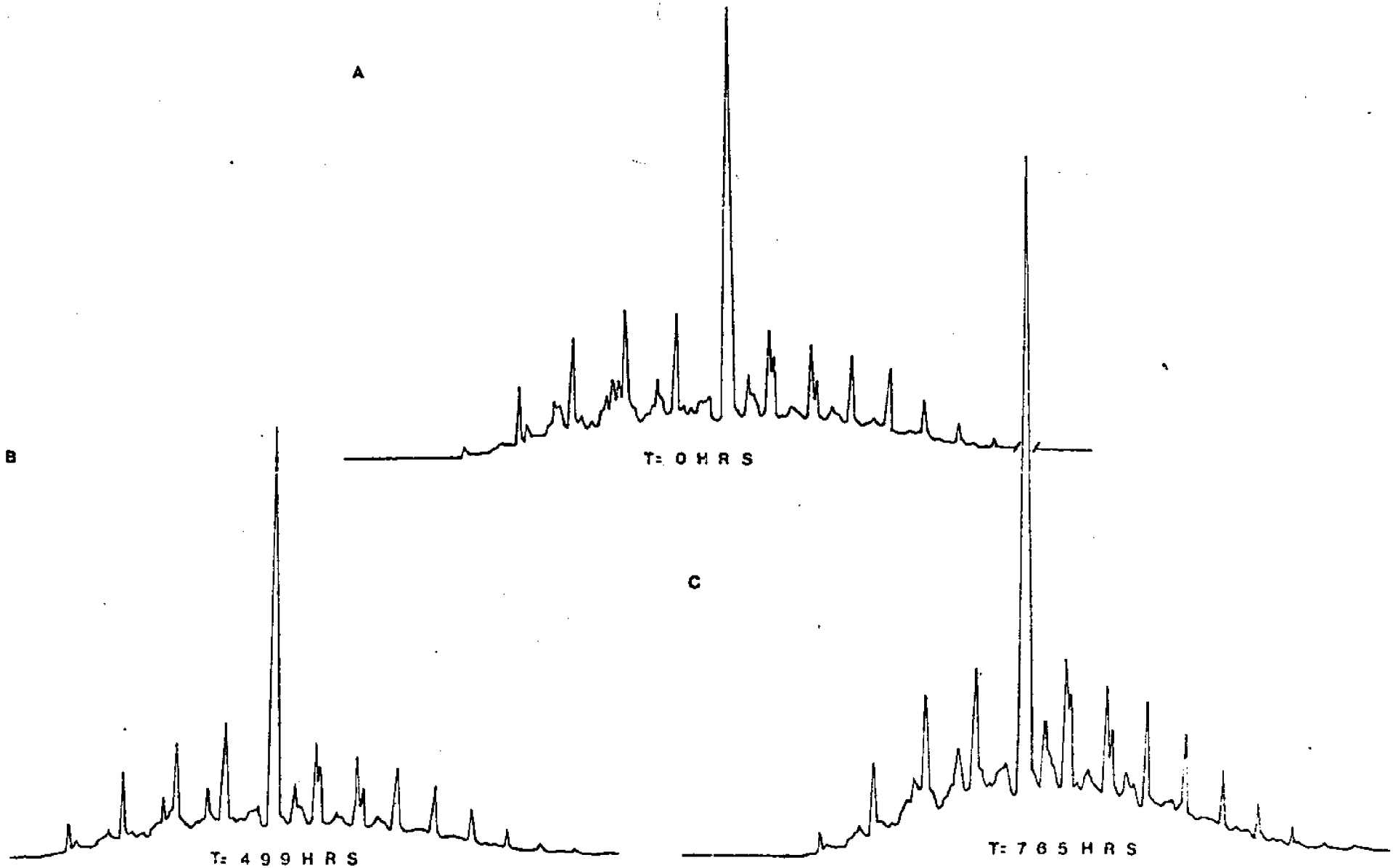


Table 8 - Peak Height Ratios From Gas Chromatographic Profiles of Diesel Oil Taken From the Oil Layer of a Continuous Culture System Containing Hexadecane in the Oil Layer (1:6 v/v Hexadecane:Oil) After Different Periods of Incubation. Systems H1 and H2 Represent the Same Experiment With Different Starting Inocula.

	Incubation Time (hrs)	n-C17/Pristane Ratio	n-C18/Phytane Ratio	n-16/Pristane Ratio	n-C20/Envelope Ratio	n-C13/Envelope Ratio
System H1	0	1.17	1.29	3.93	0.88	1.48
	499	1.20 (2%)	1.32 (2%)	3.87	0.87 (1%)	1.43 (3%)
	765	1.23 (5%)	1.29 (0%)	3.18	0.94 (6%)	1.15 (23%)
System H2	0	1.13	1.29	3.47	0.78	1.62
	529	1.09 (4%)	1.27 (2%)	3.23	0.80 (2%)	1.32 (18%)
	987	1.14 (0.8%)	1.26 (2%)	3.46	0.63 (19%)	1.11 (31%)

The hexadecane/Pristane ratio was different for each experiment. In experiment H1 the ratio dropped 19% by the end of the experiment thus indicating a significant degradation of hexadecane. In experiment H2, there was no sigificant degradation (less than 1% change). This was probably the result of differences in the inocula.

A comparison of the effluent oil profiles from H1 and H2 with those of X1 and X2 effluents (figures 6 and 9) show that the addition of hexadecane to the oil layer inhibited degradation of then-saturate series. The profiles again are skewed, thus indicating possible preferential utilization of the lower boiling fraction. The n-C13/Envelope and n-20/Envelope ratios (table 9) substantiate a decreased amount of degradation in the alkane series.

Although the total cell numbers increased to the same level seen in the unsupplemented amended systems, an enrichment for certain morphological types was not apparent at anytime. The population remained very heterogeneous during the entire experiments with very little indication of a predominant species.

Generally speaking then the addition of hexadecane does not appear to significantly affect the degradation of the oil in the oil layer when compared to the unsupplemented experiments. It does however decrease the degradation of the oil residue recovered from the effluent sample. This is possibly due to a preference of the bacterial population for the more palatable substrate and hexadecane is generally considered easier to degrade than oil itself.

4. Detergents

As a preliminary start to determine the effects of detergents on the degradation process in continuous culture, a nonionic polyoxyethylene mixture (Tween 80) was selected because of its water solubility and nontoxic nature. It is a well known industrial emulsifier and dispersing agent. It was added to the reservoir at an 0.1% concentration. For all practical purposes it had the same effect as adding glucose or propionic acid. It served as an excellent carbon source for bacterial growth and completely inhibited the oil degradation process. As seen with the other supplemented experiments, a single predominant species was enriched presumedly forcing all the oil degrading bacteria to be washed out. The detergent also caused a slight emulsification of the oil but no degradation as measured by chemical analysis.

5. Discussion

The addition of small amounts of readily degradable organic material appear to readily upset the degradation process we have observed in continuous culture. The problem with the continuous systems, however, is that once an organism is out competed for its growth substrate, it is washed out of the system. In nature the same competition would result but the washout process would not be as extreme a form of elimination. Also in nature, the place of degradation would be constantly reinoculated with fresh bacteria whereas this is not the case in continuous culture. To compensate for this discrepency we attempted to periodically reinoculate our continuous culture systems with fresh Lake Ontario water. This however did change the results sited above.

Figure 9 - Gas Chromatographic Profiles of Diesel Oil Extracted From the Effluents of Continuous Culture Systems Containing Hexadecane in the Oil Layer.

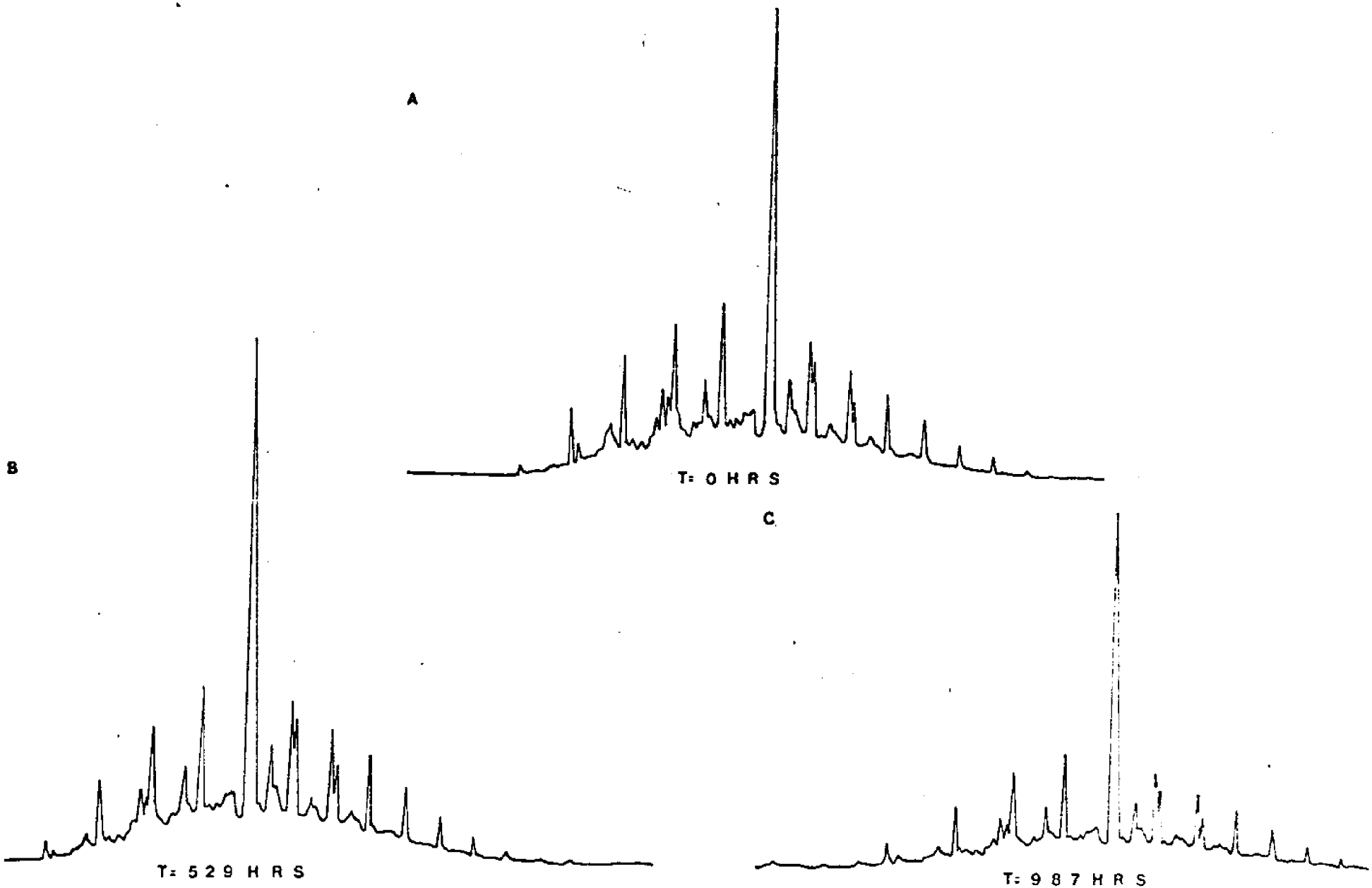


Table 9 - Peak Height Ratios From Gas Chromatographic Profiles of Oil Extracted From the Effluents of Continuous Culture Systems Containing Hexadecane in the Oil Layer.

	Incubation Time (hrs)	n-C17/Pristane Ratio	n-C18/Phytane Ratio	n-C16/Pristane Ratio	n-C20/Envelope Ratio	n-C13/Envelope Ratio
	0	1.17	1.29	3.93	0.88	1.-8
ystem H1	258	0.95 (18%)	1.12 (13%)	2.89	0.56 (36%)	0.56 (62%)
	499	0.91 (22%)	1.12 (13%)	2.23	0.79 (10%)	0.68 (27%)
	0	1.13	1.29	3.43	1.78	1.62
	529	0.84 (25%)	0.93 (22%)	2.16 (37%)	0.56 (86%)	---
ystem H2	987	0.99 (12%)	1.15 (10%)	2.69 (22%)	0.74 (54%)	---

We feel, from the results of these supplementation experiments, that the oil degradation process is very sensitive one whose efficiency is directly related to the environmental conditions present. It can be suggested at this point, until further information is available, that oil degradation in a polluted environment may be considerably slower than in an unpolluted situation. With further experiments we may be able to quantitate this relationship.

VII. Development of Bioassay System to Test Toxicity of Oil Degradation Products

A. Introduction

The active part that bacteria play in oil pollution is well recognized and is being intensively investigated thru a multitude of approaches (13). However, one area of the microbial degradation of crude oil that has not been examined in any detail is the fate of the degradation products of the oil and their ultimate ecological impact. Numerous reports in fact have elluded to the possibility that microbial degradation products of crude oil and petroleum hydrocarbons represent toxic elements which in fact could be more toxic than the oil itself (12). Of primary concern in this situation is not so much the readily obvious toxic effects but instead the more subtle, incidious long term toxic effects.

Parts of our program to study the microbial degradation of hydrocarbons is to begin to contribute some information to this central problem, i.e. are the degradation products of oil toxic to the environment and if they are, what controls their production and what is their eventual fate in the environment. To approach this problem it was first necessary to develop a suitable bioassay system. Two primary factors were considered in the development of the bioassay systems.:

a). The type of bioassay systems employed are very critical to a toxicological evaluation since it is important that even the most subtle aspects of toxicity be recognized. Many workers have maintained that if outright killing does not occur then no ecological damage has been incurred by the oil pollution. Several studies have now shown that killing is not necessarily a major criteria of toxicity but in fact more subtle types of poisoning have occurred. Blumer (11), for example, has shown that oysters and other shell fish concentrate petroleum hydrocarbons internally without "apparent" injury to the organism. The consumption of these polluted shellfish by man does, however, represent a danger, especially since carcinogenic compounds may be present. This form of potential toxicity would not have been recognized by normal bioassay procedures but instead required a sophisticated chemical detection of the oil.

In a similar manner, Kittredge (51) has shown that extremely small concentrations of water soluble components of crude oil interfere with chemical communication in fiddler crabs. This sublethal physiological effect of crude oil has divulged a whole area of toxicology which has not been considered in the past and must now be acknowledged as the primary emphasis in the development of bioassay systems. If they are going to be used to fully assess the toxicological aspects of oil pollution.

b). Toxicological studies invariably concentrate on using a single bioassay system; that is,one particular group of plants or animals. The information gained is generally important but does not have application for the total ecosystem. However, in recent years, especially as a result

of thermal pollution, large total ecosystem studies Insitu have been pursued with the idea of determining the environmental impact of a pollutant on all aspects of the community (7). These studies are supplying valuable toxicological information but they are restrictive in terms of the large outlays of time and money.

As a compromise between the single organism approach and the insitu approach, the study of several important members of an ecological community simultaneously in the laboratory can often generate highly meaningful toxicological information which can be used to access the ecological impact of a pollutant quite accurately. This multiple organism approach has several advantages; 1) it prevents an unbiased evaluation of a toxic pollutant, since several organisms are being observed and tested simultaneously, 2) it allows examination of toxic effects at all stages of a life cycle, 3) it permits a more detailed investigation into the nonlethal aspects of toxicity as they effect one group of animals and not another, and 4) by knowing the detailed developmental cycle of each organism and the environmental factors which effect their ecology, the more subtle aspects of toxicity can be observed on a community wide basis. Consequently, in our work several bioassay systems have been chosen. These include:

a) The feeding behavior of zooplankton; the test organism selected is Daphinia magmum and the bioassay will focus on the animal's ability to find food and the rate at which food is assimilated using radioactive algal food sources.

b) The embryological development of fish; the test organism selected is the Japanese Medaka, Oryzias latipes, and the bioassay will focus on TL 50, and abnormalities of organ development at various stages during embryological growth.

c) Cytotoxic effects on animal cells; the test organism selected is a tissue culture line of rat skin cells and the bioassay will focus on the production of gross cellular and mitotic anomalies such as amitosis, micronucleation, and multiple cytokinesis.

d) The growth and photosynthesis of algae; the test organism selected is Chlamydomonas reinhardtii and the bioassay will focus on the effects on growth both autotrophically and heterotrophically and on the rates of photosynthesis.

All of the above bioassay systems are presently under development and appear to be potentially useful toxilogical tools. In each assay we are concentrating on three sources of toxic materials:

a. water extracts of chemically pure hydrocarbons (namely toluene)

b. water extracts of diesel oil

c. effluents from continuous culture systems involving microbial degradation of oil source of degradation products.

To date, the most highly developed bioassay system is with the Japanese Medaka and we will report only on this system. This work has been in collaboration with Dr. Terry Haines and his graduate student, Frederick Stoss.

B. Methods

1. Care and Handling of Adult Fish

Fish in a sexually mature condition, were obtained from Carolina Biological Supply Company and kept in a 20 gallon aquaria at room temperature. From this initial population a large stock culture was raised from which the eggs for testing were obtained. Adults were raised in ratio of 6 females to 40 males for every 20 gallons of water. Culture water was constantly checked for pH (7.2 to 8.2), alkalinity (96± 10mg/l), oxygen (8.5+ 1mg/l) and temperature (24°C± 1°C). Snails and green plants were also included in the aquaria. Adults were fed live brine shrimp to insure maximum breeding potential. An artificial light environment was maintained with an equivalent 16 hour day and 8 hour night. Aquarium water was periodically treated with methylene blue to prevent fungal diseases.

2. Care and Handling of Eggs

Oocytes in the female, which had developed overnight, ovulate with the onset of light and are fertilized by the male shortly thereafter. Oviposition occurs 1 to 2 hours after the start of the light period and the spawned egg cluster can be collected either directly from the female or from the vegetation where they are frequently deposited by the female. Eggs were washed with distilled H_2O, separated with a forceps, and then incubated in sterilized embryo rearing medium of Kircher and West (33).

3. Embryonic Development

The Medaka follows typical teleostean patterns of development. The stages of development are shown in Table 10.

4. Preparation of Toxic Solutions

Water extracts of toluene were prepared by layering 1 ml of toluene on the surface of 250 mls of sterile rearing medium and gently stirring the rearing medium with a magnetic stirrer (100 rpm) for 24 hours at 23°C. These conditions gave maximal extraction without emulsification. The water soluble fraction was termed the 100% toluene-water extract.

The concentration of toluene dissolved in the water was determined by ultraviolet light absorbtion at 260 nm. Values were determined relative to a standard curve using carbon tetrachloride as the solvent. The method was sensitive to a range of toluene concentration from 866.9 mg/l to 21.75 mg/l. 100% toluene-water extracts contained 164 mg/l of toluene.

Table 10 - Embryonic Stages in the Development of the Japanese Medaka.

Stage Number	Approximate Age	Description
Stage 1	0.0 hrs.	oviposition, unfertilized egg
Stage 2	0.2 hrs.	fertilized egg
Stage 3	0.8 hrs.	germinal disc
Stage 4	1.5 hrs.	2-cells
Stage 5	2.0 hrs.	4-cells
Stage 6	2.5 hrs.	8-cells
Stage 7	3.0 hrs.	16-cells
Stage 8	3.5 hrs.	32-cells
Stage 9	4.0 hrs.	64-cells
Stage 10	6.0 hrs.	early high blastula
Stage 11	9.0 hrs.	late high blastula
Stage 12	12. hrs.	flat blastula
Stage 13	13. hrs.	dorsal lip gastrula
Stage 14	15. hrs.	embryonic shield
Stage 15	17. hrs.	mid gastrula
Stage 16	20. hrs.	late gastrula
Stage 17	23. hrs.	early neurula
Stage 18	26. hrs.	late neurula
Stage 19	29. hrs.	"Blastopore"
Stage 20	33. hrs.	anterior somites
Stage 21	36. hrs.	pericardial cavity, optic cup
Stage 22	40. hrs.	optic lens & otocyst
Stage 23	46. hrs.	heartbeat
Stage 24	52. hrs.	circulation
Stage 25	54. hrs.	body movement & otoliths
Stage 26	56. hrs.	retinal pigmentation
Stage 27	62. hrs.	pectoral fin bud
Stage 28	74. hrs.	pink blood
Stage 29	84. hrs.	vitelline veins sinuous
Stage 30	102. hrs.	urinary bladder
Stage 31	121. hrs.	pectoral fin movement & caudal fin
Stage 32	128. hrs.	liver rudiment
Stage 33	144. hrs.	swim bladder
Stage 34	168. hrs.	jaw movement & yellow coloratio
Stage 35	200. hrs.	spleen and mouth
Stage 36	264. hrs.	hatching

Water extracts of diesel oil were prepared in a similar manner but allowed to extract for a total of 72 hours. Embryo rearing medium was used as the extracting solution. A gas chromatographic profile of a pentane extract of water extract is shown in Figure 10.

Bacterial degradation products were collected directly from the continuous culture systems in which diesel oil was being degraded. Effluents were collected, centrifuged, filter sterilized and frozen to screw cap plastic bottles until needed. No attempt has been made to characterize or quantitate the degradation products in the effluent.

5. Procedure for Bioassays

Bioassays were conducted using 100%, 75%, 50%, 25% and 0% of the toxic solution diluted in sterile rearing medium. These bioassays were modifications of standard procedures outlined by Hart et al (26), Doudoroff et al (16) and A.P.H.A. (3).

Fertilized eggs were taken from the female's abdomen within two hours after fertilization and reared until the desired stage of development. Stages were selected that represented: a) early cleavage before the chorion had become water hardened b) mid cleavage c) late cleavage d) 1-day old blastula e) two day old embryo f) three day old embryo g) 7-9 day old embryo h) prehatching embryo of 9-10 days i) newly hatched fry of 10-11 days in age and j) fry one week old.

Groups of 10 embryos at the same stage were transferred to sterilized cylindrical screw-top glass vials containing 21 ml of test solution. These vials minimized any volitalization of the toxic materials. Under these conditions control embryos survived for at least six weeks.

For each vial, mortalities, deformities and organ development were monitored. Mortalities were recorded at 24, 48, and 96 hours after exposure to the toxic materials. For young undifferentiated eggs, death was accompanied by the uptake of methylene blue and the swelling of the eggs. For young fry and older eggs, death was defined as a discontinuation of heart beat. For mature fry, death was defined as the abolishment of respiratory and body movements and the lack of response to mechanical stimuli.

Medium tolerance limits (TL 50) were determined for each stage of development using the concentration of toluene allowing 50% survival for that test period.

C. Results and Discussion

1. Mortality Effects of Toluene

The toxicity of water extracts of toluene was determined for various stages of embryonic development and the results are shown in Table 11 and Figure 11. The TL 50 values represent the median survival rate of the embryos measured in mg/l of toluene.

Figure 10 - Gas Chromatographic Profile of Hydrocarbons Extracted From Diesel Oil with Water.

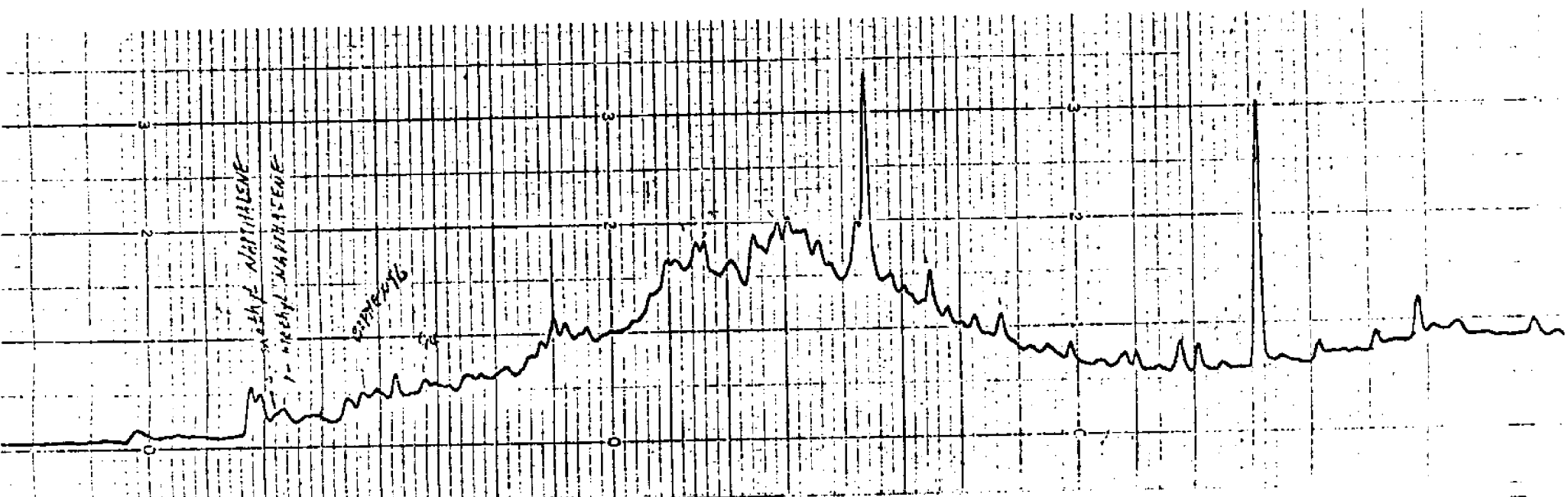
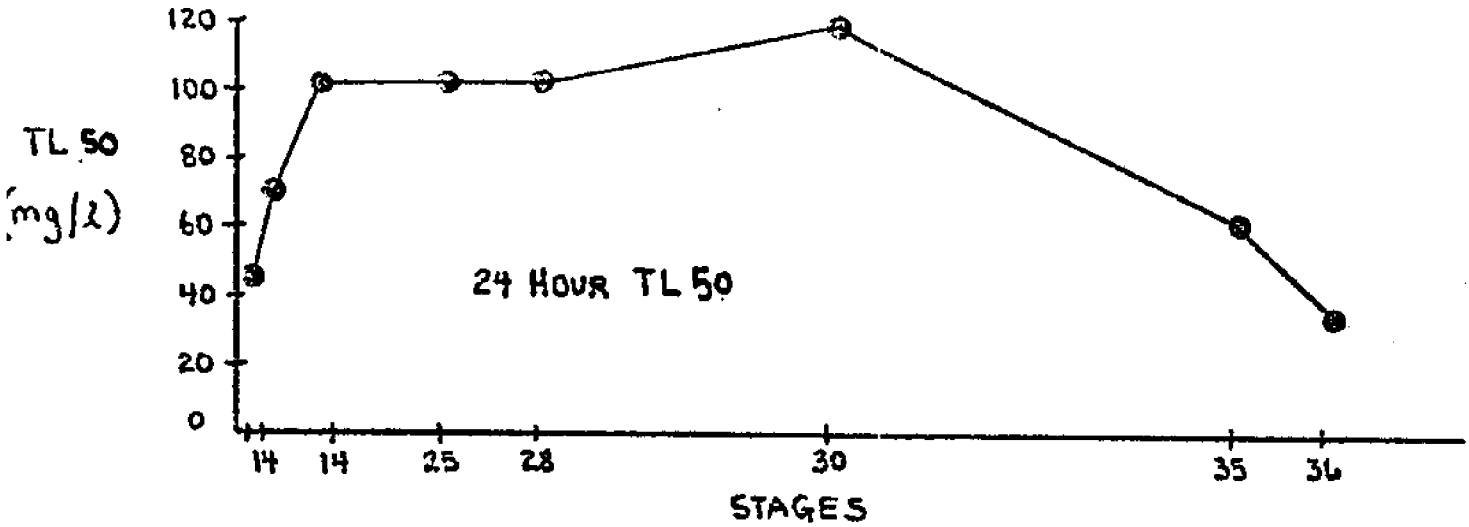
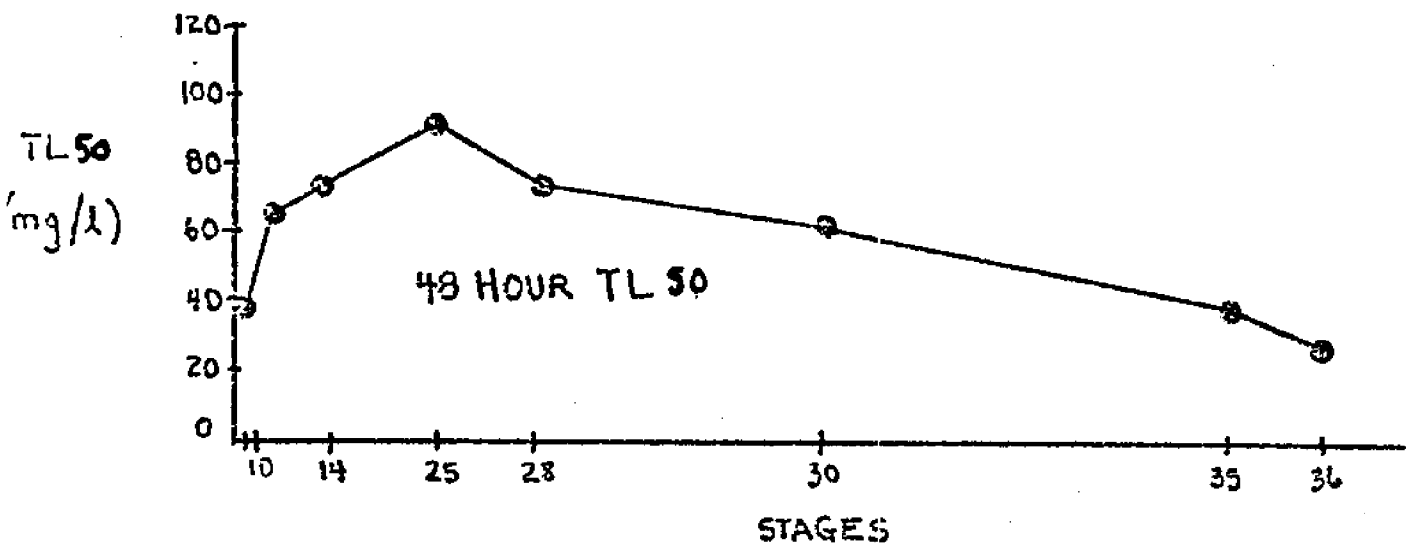


Figure 11 - The Effects (as TL 50) of Water Extracts of Toluene on Medaka Embryos.





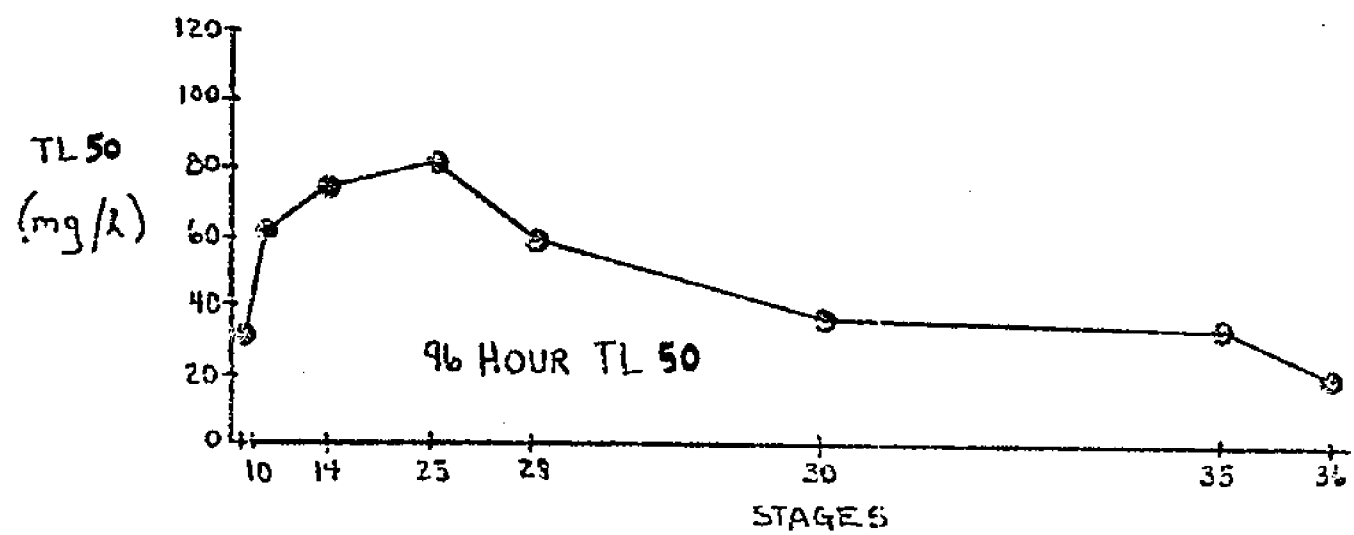
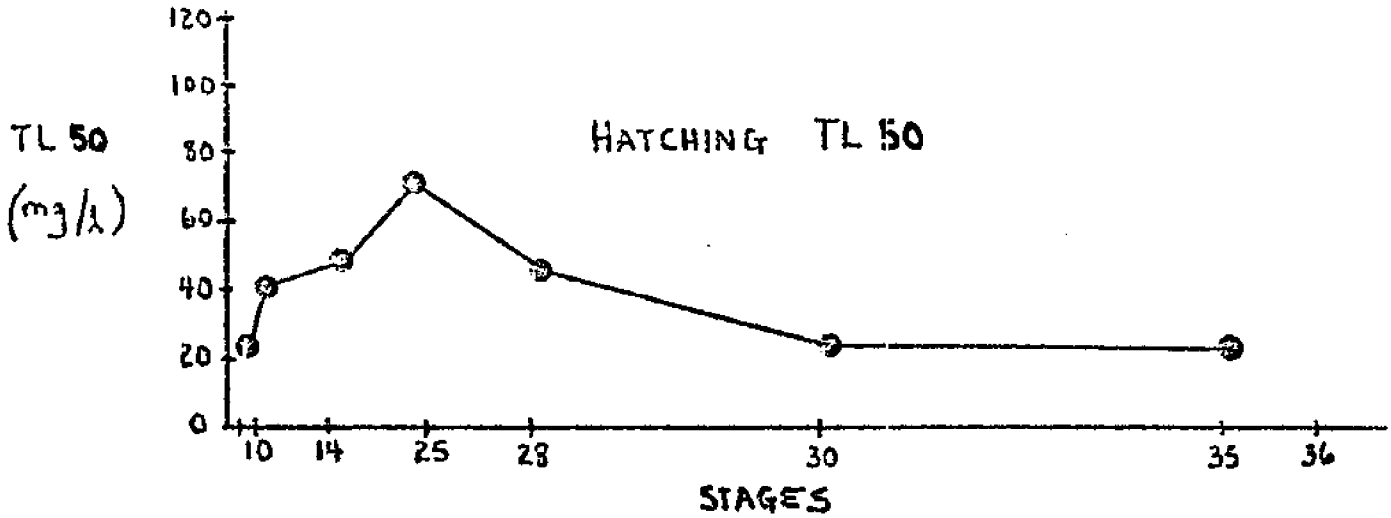
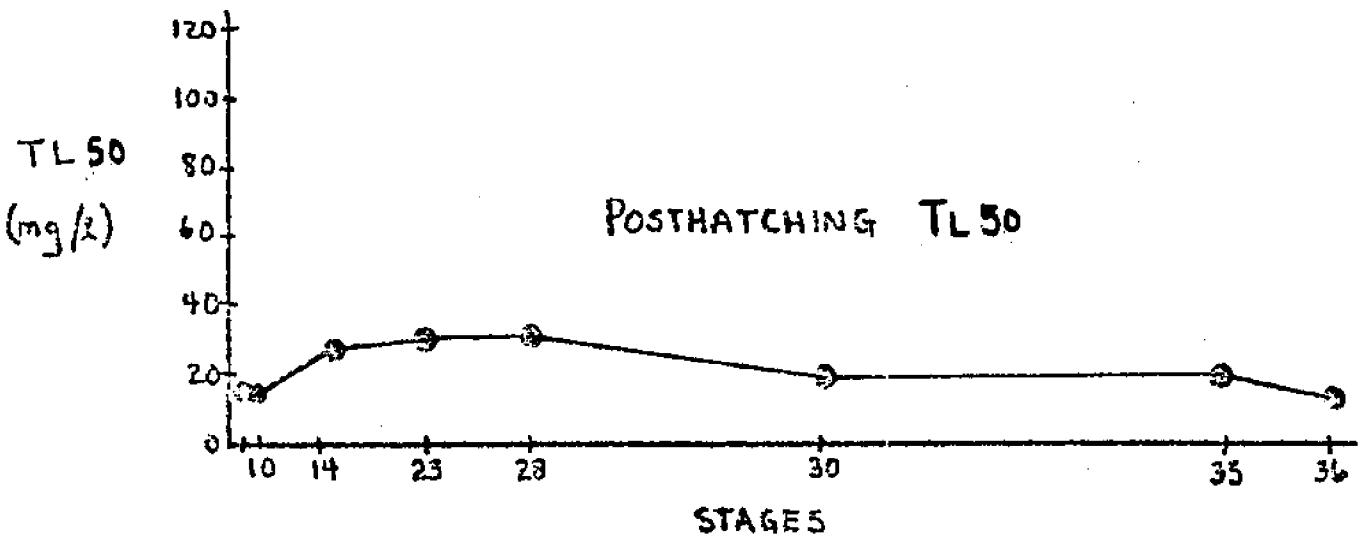


Figure 11. continued.





The results indicate that the maximum TL 50 values were between the second and sixth day of development and the minimum TL 50 values occurred during the early cleavage stages (i.e., before 4 hours of development). The pattern of resistance to toluene is shown in Figure 11. As can be seen the embryos are the most sensitive at the early stages of development (1-14) but become increasingly more resistant as development proceeds up to the sixth day (stage 33). Beyond six days, the resistance again drops reaching a low point upon hatching (stage 36). The newly hatched fry were also quite sensitive to the toluene. Thus, it is concluded that early cleavage stages and hatching stages of development were the most sensitive.

Mortalities were also a function of the exposure time to the toxic solution (Table 11 and Figure 11); generally the longer the exposure to the toxic material, the greater the possibility of lethal effects.

2. Developmental Defects Induced by Toluene

Table 12 summarizes the teratogenic effects to the Medaka. The results indicate that the production of deformities depends upon the concentration of toxic solution, the duration of exposure and the stage of embryonic development. Normal development occurred at all concentrations of toluene below 16.42 mg/l. Increasingly more abnormal development appeared as the concentration of toluene increased. Generally, the earlier stages were the most susceptible and increasing the exposure time increased the number of deformities.

The types of deformities observed varied greatly but were readily reproducible and predictable under similar conditions. The severity of the deformity was a direct function of the toluene concentration.

The developing heart and vitelline circulatory system were the most sensitive organ systems. The heart usually never developed beyond a degenerate pulsating vessel lacking all traces of typical flexures and chambers. The circulation system was stunted and resulted in pink blood islands which formed near the oil droplet in the egg. Disturbances to the vascular system accounted for early death of the embryo.

Deformities of the tail region were also commonly observed. Tails were shortened or flexed to the left or right and sometimes notochord displacements were also observed. Embryos with flexed tails that survived hatching, were incapable of normal swimming movements and generally died several days after hatching.

3. Effects of Water Soluble Extracts of Diesel Oil

Using the toluene data as a background, water extracts of diesel oil were tested for their toxicity in the same manner used to test toluene. Table 13 shows the media tolerance limits of such an extract as a function of the stage of development. As can be seen, the results mimic the toluene experiments; early and late stages of development were the most sensitive while middle stages were relatively resistant. Similar teratogenic effects were also noted. Preliminary gas chromatographic analysis showed aromatic

Table 11 - TL_{50} (mg/l) for the Medaka Eggs Treated With the Water Extracts of Toluene for Varying Periods of Time.

Stage	Type of Development	24 hr	48 hr	96 hr	(hatching) 11 day	(post hatching) 18 day
5	early cleavage	44	39	33	23	16
8	late cleavage	82	82	79	46	--
8	" "	61	48	43	33	16
9	" "	62	62	62	41	--
10	blastula	110	100	100	65	--
11	" "	123	41	41	16	--
11	" "	69	64	57	48	25
12	" "	110	103	94	71	--
19	" "	97	67	62	33	--
25	2 day old	102	90	80	74	--
27	3 day old	113	100	80	74	46
28	4 day old	108	46	39	25	--
28	" "	85	72	59	41	21
30	6 day old	102	41	41	33	--
30	" "	135	82	32	18	--
35	9-10 day old	61	39	35	26	20
36	11 day old (fry)	36	36	21	--	13
36	" "	48	21	13	--	10
36	" "	26	26	26	--	21

Table 12 - Teratogenic Effects of Different Concentrations of Toluene to Medaka Embryos After 48 Hours of Exposure to the Toxic Solution.

Stage Exposed	Concentration (mg/l)				
	164.25	123.14	82.13	41.06	16.43
Early cleavage	+	+	+	+	-
Mid cleavage	+	+	+	+	-
Late cleavage	+	+	+	+	-
Blastula	+	+	+	+	-
2 day old	+	+	+	-	-
3 day old	+	+	+	-	-
4 day old	+	+	-	-	-
6 day old	-	-	-	-	-
9-10 day old	-	-	-	-	-
11 day old	-	-	-	-	-

+; indicates abnormalities detected

-; indicates abnormalities not detected

Table 13 - Effects of Water Soluble Extracts of Undegraded Diesel Oil on Embryological Development of Oryzias latipes, Japanese Medaka.

Stages at Which the Embryo was Introduced into the Extract Solutions	Median Tolerance Limit (measured as percent of extract)	General Remarks
11	30%	Blastomeres fail to differentiate, severe growth retardation.
12	36%	Circulation disruption
13	29%	Growth retardation; Circulation disruption.
20	75%	Retarded growth; eye pigment distribution not normal; circulation disruption.
21	63%	
30	38%	Irregular heart rate; died during hatching or shortly after.
35	18%	Irregular heart rate; died during hatching; sluggishness and disoriented swimming if survived hatching.
36	15%	Sluggishness; disorient swimming movements.

hydrocarbons present in the extract at concentrations comparable with those in the toluene extraction. Extractions from topped oil had slightly less toxicity than raw diesel oil.

4. Effects of Bacterial Degradation Products of Oil

Effluents from the continuous culture systems described above, in which diesel oil was attacked by bacterial populations from Lake Ontario, were collected periodically and filter sterilized. These sterile effluents were then diluted in Medaka embryo rearing medium and tested for toxicity as described for toluene. The results of this work are shown in Table 14. Even though this is only a preliminary study, it would appear that several tentative conclusions can be drawn.

a) The earlier stages of development (stages 10-20) are the most sensitive to bacterial degradation products.

b) In stages 10-11, it appeared that after 10 days of degradation, the oil was less toxic than water extracts of undegraded oil. However, after 23 days of incubation with the bacterial population, the degradation products of the oil appeared to become more toxic, perhaps even surpassing the toxicity of water extracts of undegraded oil.

c) In stages 16-20, the degradation products were always more toxic than the water extracts of undegraded oil and, if anything, appeared to increase in toxicity as degradation progressed. Toluene at these stages was much less toxic.

d) In stages 29-35, degradation products were generally less toxic and appeared to be increasingly less toxic with increased degradation time.

Experiments with newly hatched fry have shown that the bacterial degradation products were particularly toxic at this stage. Generally, about 3-4 days after hatching, at a time when the fat droplet or oil globule was absorbed, 90-100% of the fry were killed outright. This lethal effect was not observed with toluene or water extracts of undegraded oil.

5. Discussion

The Japanese Medaka Oryzias latipes, appears to be an excellent biological assay for toxicity studies. The fish produce large quantities of eggs consistently, the embryological events are well worked out and readily observable and their sensitivity to hydrocarbons is high and experimentally reproducible. The bioassay system is also very manageable and does not require specially trained technicians or expensive equipment. Although all the studies reported here were performed under closed conditions, the bioassay system appears readily amenable to flow-thru studies.

Table 14 - Effects of Effluents From Continuous Culture Systems Involving the Bacterial Degradation of Diesel Oil on the Embryological Development of the Japanese Medaka.

Stages at which the embryo was introduced into effluent solution	Length of bacterial degradation (hr)*	Median tolerance limit measured as the per cent of effluent	General Remarks
11	67	60%	General disruption of circulation; slight eye malformation.
29	67	35%	Sluggishness and slowed irregular heart beat; died during hatching.
34	67	30%	Extreme sluggishness; irregular heart beat; disoriented movement after hatching.
8	256	40%	Growth retardation; disruption of circulation patterns.
11	256	58.3%	Disruption of circulatio.
12	256	40.1%	Disruption of circulatio slight tail bending.
18	256	25%	Slight tail bending; growth retardation; slowed irregular heart rates.
28	256	50%	Irregular heart rates; died during hatching.
29	256	25%	Sluggishness; died durin hatching.
30	256	25%	Sluggishness; died durin hatching.

Stage	Degradation (hr)*	TLm	General remarks
35	256	30%	Sluggishness; died during hatching.
36	256	30%	Sluggishness; disoriented swimming movements.
10	269	30%	Disruption of circulation.
16	269	40%	Disruption of circulation; slow irregular heart rates.
32	269	20%	Sluggishness; died during hatching.
29	500	81%	Sluggishness; irregular heart rate.
30	500	50%	Sluggishness; died during or shortly after hatching.
35	500	50%	Sluggishness; irregular heart rate; died during or after hatching.
36	500	35%	Sluggishness; disoriented swimming movements.
8	570	38%	Retardation of growth; disruption of circulation; eye pigment distribution not normal.
10	570	25%	Slowed irregular heart rates; circulation pattern disrupted
18	570	20%	Circulation patterns disrupted.

*Measured from time of inoculation of continuous culture system.

Water soluble components of oil and toluene were found to be toxic as expected. However, it is clear that with the detailed study of embryological development, all stages are not equally susceptible to the toxic material. In the case of toluene and the water extracts of undegraded oil, the embryos become more resistant as the chorion of the eggs became water hardened (late early and middle stages). Thus it appears very useful to investigate the toxicity of a material at each particular stage of embryonic development rather than study one aspect of the animal's life cycle.

The toxicity of the bacterial degradation products also proved interesting and again showed the advantage specific of stage-oriented studies. At stages where toluene and water extracts of undegraded oil were generally the least toxic, the bacterial degradation products of the oil were most toxic, i.e., the stages where the chorion hardens and where the newly hatched fry engulf the oil droplet. The extent of bacteria degradation of the oil was also important, causing less toxicity in some cases and more in others. It therefore, appears possible that changes in toxicity during the degradation may signal a definite change in the composition of the oil or the onset of the production of a different product from the degradation process. This could be due to the sudden availability of some component in the oil or a sudden change in the bacterial population. Experiments are presently underway to study these possibilities.

VIII. Conclusions and Recommendations

It can be concluded from this work that the use of continuous culture systems to study oil degradation is a productive approach which has and will generate information which can be used in evaluating and acting upon natural oil pollution situations. Because our results are very similar to actual observations in the field, it appears reasonable to make recommendations based on these results. Our conclusions and recommendations are as follows.

A. The natural oil degradation process is the same regardless of whether it takes place in soil, in sediment or in aquatic bodies of water. The results presented in this report have shown that the water insolubility of oil dictates that it invariably break up into small droplets, regardless of its location, and that the primary bacterial attack is on these droplets. The attack occurs on the outside of these droplets and moves inward and the length of the overall degradation process will depend on the volume of the drop.

The primary attack on these oil droplets is the most critical aspect of the entire degradation scheme. Its initiation occurs only if the oil droplets are allowed to come in contact with a specific group of hydrocarbon degrading bacteria which specialize in attaching to the oil and eventually impregnating it.

We recommend, consequently, that any clean up procedure for oil pollution be based on optimizing this initial attachment and impregnation process. This can be attained most satisfactorily by simply mechanical dispersion of the oil to enhance the formation oil droplets and their contact with bacterial populations. When an oil spill on a freshwater lake is involved, the oil should be contained (using physical barriers where necessary) to allow either natural dispersion to take place (wave action, wind, bacterial emulsification, etc.) or the implementation of mechanical dispersion procedures (spreaders, mixers, agitators, etc.).

B. We do not recommend the use of chemical dispersants, such as detergents, since they intefere with the primary bacterial attack on the oil droplets. At the very least, these chemicals seem to potentially serve as an alternate source of carbon and energy for microbial growth. This growth will consume the available sources of nitrogen and phosphorous and thereby greatly slow down the oil degradation process.

C. Since all of our degradation studies have required the addition of substantial amounts of inorganic nitrogen and phosphorous, above that found in Lake Ontario water itself, we recommend that inorganic fertilizers be applied to areas of oil pollution to enhance the natural degradation process. Pilot studies should be initiated to determine the amount to be applied for each type of environmental situation involved.

D. The removal of oil from the surface of a body of water does not constitute total degradation of the oil. It reflects instead, an important initial attempt by bacteria to emulsify the oil and chemically modify it to a small degree. These two steps are responsible for setting up conditions which greatly enhance the degradation process at succeeding stages; without them further degradation is slower and incomplete.

We recommend, therefore, that anyone involved in oil pollution problems be educated to the fact that any man-made attempt to speed up the disappearance of the oil (chemical dispersants, sinking procedures, etc.) may in fact, circumvent these initial actions by bacteria and result in a partially degraded product of oil which is more recalcitrant or persistent than the oil itself. In other words, from the results in this study, it is clear that the initial set of events in microbial oil degradation greatly determines its fate in an aquatic environment.

E. We also conclude from our results, that oil in organically polluted aquatic environments may, in fact, undergo a much slower rate of degradation. This is due to the availability of organic materials which are preferred by bacteria over oil and petroleum hydrocarbons as a source of carbon and energy. The only recommendation we can offer at this time is to supplement the polluted areas with sources of nitrogen and phosphorous to assure that there is enough inorganic nutrient remaining for use by the oil degrading bacteria.

F. The application of oil degrading bacterial seed cultures to oil spills as a means of increasing oil removal and degradation is a worthless concept except in very specialized situations. These seed cultures are undoubtedly outcompeted by natural bacterial populations; they, at the most, only emulsify the oil without any degradation, and they quite possibly may interfere with the natural degradation processes thereby increasing the residence time of the oil in the aquatic environment. It is also quite clear that oil degradation is a very complex process which cannot be readily initiated by simple mixtures of hydrocarbon degrading cultures.

G. Finally, there are still many unknown factors in oil degradation which have not been detailed but which are desperatly needed if sound judgements are going to be made about oil pollution policies. Continuous culture appears to offer an approach for obtaining this information without massive, expensive, and dangerous field experiments. It would appear at this time that one of the most important areas is to determine the actual fate of the oil once it goes through this primary attack by bacteria described herein. We are presently testing sequential continuous culture systems to obtain this information.

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